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# A direct and indirect mechanism for CCR5 in morphine and HIV-1 mediated neurodegeneration

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A direct and indirect mechanism for CCR5 in opioid and HIV-1 mediated  
neurodegeneration

A dissertation submitted in partial fulfillment of requirements for the degree of Doctor of  
Philosophy at Virginia Commonwealth University

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### **List of Abbreviations**

AIDS	Acquired immunodeficiency syndrome
ANI	Asymptomatic neurocognitive impairment
BBB	Blood-brain barrier
cART	Combined antiretroviral therapy
CCR5	C-C chemokine receptor 5 (CD195)
CCR5-Δ32	C-C chemokine receptor 5 with 32 base pair deletion
CD4	Cluster of differentiation 4
CNS	Central nervous system
CXCR4	C-X-C chemokine receptor 4 (CD184)
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (CD209)
DOR	Delta opioid receptor (δ-opioid receptor)
DOX	Doxycycline hyclate
GFAP	Glial fibrillary acidic protein
gp120	Glycoprotein 120
HAART	Highly active antiretroviral therapy
HAD	HIV-1 associated dementia
HAND	HiV-1 associated neurocognitive disorders
HIV-1	Human immunodeficiency virus type-1
HIVE	HIV-1 encephalitis
HSPGS	heparin sulfate proteoglycans
Iba-1	Ionized calcium binding adaptor molecule-1
KOR	Kappa opioid receptor (κ-opioid receptor)
LRP	Low density lipoprotein receptor-related protein

MAP2	Microtubule associated protein-2
MCMD	Minor cognitive/motor disorder
MCP-1	Monocyte chemoattractant protein-1
MIP-1 $\alpha$	Macrophage inflammatory protein-1 alpha (CCL3)
MIP-1 $\beta$	Macrophage inflammatory protein-1 beta (CCL4)
MOR	Mu opioid receptor ( $\mu$ -opioid receptor)
MVC	Maraviroc
NF- $\kappa$ B	Nuclear factor kappa B
NMDAR	N-methyl-D-aspartate receptor
RANTES	Regulated upon activation normal T-cell expressed and secreted (CCL5)
R5-tropic	CCR5-tropic
STD	Sexually –transmitted disease
Tat	Transactivator of transcription
X4-tropic	CXCR4-tropic

## **Abstract**

### **A DIRECT AND INDIRECT MECHANISM FOR CCR5 IN OPIOID AND HIV-1 MEDIATED NEURODEGENERATION**

By Elizabeth M. Podhaizer, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2014

Major Director: Kurt F. Hauser, Ph.D., Professor of Pharmacology & Toxicology

Human immunodeficiency virus (HIV)-1 infection currently affects over 34 million people worldwide, and despite the use of cART, the prevalence of HIV-1 associated neurocognitive impairments (HAND) has not declined. Additionally, other co-morbid factors such as the abuse of injection drugs (i.e. heroin, morphine) increase both the frequency and the speed by which patients progress to AIDS. To begin to understand the mechanisms, we chose to examine a pathway, through CCR5, which may act as a convergence point for opioids and HIV-1 proteins. C-C chemokine receptor 5 (CCR5) is an immune receptor involved in physiological processes in the brain in addition to mediating neuroinflammatory signaling events, and it is a co-receptor for HIV-1. CCR5 interacts directly with gp120 to facilitate HIV-1 infection, and may interact indirectly with HIV-1 Tat through convergent signaling mechanisms. Additionally, CCR5 is modified by opioid responses, and so may be central to opioid-HIV-1 interactions that are seen in



our model. We hypothesized that CCR5 would mediate the opioid-HIV-1 interaction. We examined both HIV-1 gp120 and HIV-1 Tat, both for interactions with opioids and modification by the CCR5 antagonist, maraviroc.

HIV-1 gp120<sub>ADA</sub> was neurotoxic on its own, but showed no interactions with morphine. However, further probing revealed that morphine can in fact modify the neurotoxic effects of gp120, but that the response is dependent on gp120 strain. We did, however, find that morphine did enhance the neurotoxicity of Tat, which we've shown previously, as well as that inhibition of CCR5 can prevent this interactive effect. Additionally, use of CCR5 knockout glia or neurons modified the response and suggests that neurons and glia play different roles in the integration of opioid and HIV-1 signals. Sublethal effects of morphine and Tat were also dampened by maraviroc pretreatment or use of knockout cells, as was the secretion of chemokine ligands.

Manipulation of CCR5 showed utility in preventing neurodegenerative effects both to HIV-1 proteins alone as well as to the interactive opioid-HIV-1 signaling responses and suggests that maraviroc, a cART therapeutic used to prevent viral entry, may also aid in reducing the chronic inflammatory state of the CNS that leads to the persistent neurocognitive complications.



## **Chapter 1**

### **Introduction to HIV-1 in the Central Nervous System**

#### **Origins of HIV-1 and its groups and clades**

HIV-1 and HIV-2, while of the same lentiviral class, are not directly related and have different profiles in terms of global distribution and the severity of the pathology. HIV-2 is derived from SIV of the sooty mangabey (Hirsch et al., 1989) and is restricted to Western Africa (de Silva et al., 2008). While the AIDS profiles of the two viruses are similar, few of those infected with HIV-2 actually progress to AIDS and few have limited neurocognitive impairment (Rowland-Jones and Whittle, 2007).

HIV-1 is classified as a pandemic, with 34 million people infected globally (UNAIDS, 2010). This virus has origins in SIV from infected chimpanzees (Huet et al., 1990; Gao et al., 1999) and is more complicated than its HIV-2 counterpart, containing four distinct lineages, designated as groups M, N, O, and P (Sharp and Hahn, 2011). Group M is the most prevalent and the group associated with the global HIV-1 problem, while N, O, and P variants are highly limited and restricted to specific locations. Group O is responsible for only 1% of the infections and is present in Cameroon, Gabon, and other countries of this region (De et al., 1990; Gurtler et al., 1994; Mauciere et al., 1997; Peeters et al., 1997); group N has only been shown to be responsible for 13 cases (Vallari et al., 2010), and group P infection has only been found in two cases, also in Cameroon (Vallari et al., 2011). The origin of the pandemic group M virus have been traced back to between 1910 and 1930 (Korber et al., 2000; Worobey et al., 2004) whose origins in Cameroon to the city known now as Kinshana in the Democratic Republic of Congo (Vidal et al., 2000). HIV-1 group M virus was able to spread as the

city, which was a major route for commerce, brought many people traveling to divergent places together (Sharp and Hahn, 2008). The mechanism by which HIV-1 spread and its replication process allowed further diversification among group M virus into nine different subtypes or clades, A-D, F-H, J, and K, which doesn't take into account recombinant forms (Taylor et al., 2008). The clades have regional specificity with B as the most prevalent, affecting the Americas, much of Europe, and Australia (Gilbert et al., 2007), and C affecting much of Africa and Asia, while the others have smaller regional distributions (Taylor et al., 2008). The clades have diverged from their common origins in such a way that the subtype of virus by which a person is infected can be used as a predictor of the severity of the pathogenicity and the subsequent neurocognitive outcomes. Clade D has been shown to be more virulent with an increased incidence of impairment and cases of AIDS (Kiwanuka et al., 2010; Sacktor et al., 2009). Clade C on the other hand generally does not show severe progression to AIDS due, potentially, to the sequence of the Tat gene (Ranga et al., 2004). Fifty to seventy years after HIV-1 emerged, it became recognized as a pandemic in need of global attention.

### **Epidemiology of HIV-1**

There are currently about 34 million people infected with HIV-1, 1 million of those in the United States, and each year 1.8 million people die from the disease, while 2.7 million are newly infected (UNAIDS, 2010). Since HIV-1 was first discovered in 1981 (CDC, 1981), close to 60 million people have contracted the virus and the death toll has reached about 25 million (Merson et al., 2008). HIV-1 is one of the most deadly diseases that the world has experienced and the largest cause of mortality in sub-

Saharan Africa (Merson et al., 2008). There are three routes by which one can contract HIV-1; it can be transferred through sexual intercourse, or by percutaneous or perinatal routes (Hladik and McElrath, 2008;Cohen et al., 2011), though it appears that sexual transmission is the main mode of spread of the virus, and because 80% of the transmission is across mucosal surfaces, HIV-1 is generally considered a sexually transmitted disease (STD). A major influence in the spread of HIV-1 is the use of injection drugs. Virus can not only be transmitted through blood-to-blood contact when needles are shared, but the exchange of sex for drugs is not an uncommon occurrence and aids in viral transmission (UNAIDS, 2010). Though areas differ in their modes of transmission of the HIV-1 virus, significant problem areas for transmission through injection drug use include eastern Europe, east Asia, South-East Asia and Latin American with rates in sub-Saharan Africa on the rise (UNAIDS, 2010). Treatment regimens for HIV-1 involving mono- or dual-therapy began in the mid 1980's, but during this time, contraction of opportunistic infections and progression to AIDS was both common and swift (Arts and Hazuda, 2012). It wasn't until the emergence of highly active antiretroviral therapy (HAART) in 1996 that significant changes to disease progression were made (Collier et al., 1996;Staszewski et al., 1996;McKean and Carlton, 1977). These drugs were composed of three inhibitors targeting different locations in the HIV-1 replication cycle and included: a nucleotide reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor, and a protease inhibitor. However, now the terminology has switched to combined antiretroviral therapy (cART) which is a more general term and encompasses other more recent therapeutic advances, including entry inhibitors and fusion inhibitors. Under either name, HAART or

cART, combination therapy has significantly altered the course of the disease, changing it from a disease through which people would die in a matter of months, to a manageable chronic illness where people are now living into their second decade (Dieffenbach and Fauci, 2011;Palella, Jr. et al., 1998;Ray et al., 2010).

### **Need for Continued Improvement to cART Therapy**

Although cART has significantly influenced HIV-1 outcome, the extended life-span of infected individuals has unmasked problems associated with long-term infection. CNS effects of HIV-1 are collectively known as HIV-1 associated neurocognitive disorders (HAND) and range from the least severe, asymptomatic neurocognitive impairment (ANI) to minor cognitive/motor disorders (MCMDs), to the most severe form known as HIV-1-associated dementia (HAD) (Ellis et al., 2007). While the profile of the disease has shifted, in that the incidence of HAD has decreased from ~18% to ~8% following implementation of cART therapy, the less severe forms of HAND have concomitantly increased and still affect 40-60% of HIV-1 infected people (Cysique et al., 2004;Ellis et al., 2007;Sacktor et al., 2002) (Figure 1.1). In addition, CNS pathology associated with HIV-1 infection still persists despite cART therapy. The impairments that are noted are highly subcortical, with areas of the basal ganglia and hippocampus predominantly affected (Nath et al., 2000). This correlates with areas of higher viral load, suggesting a discrete mechanism by which HIV-1 is able to target these brain regions. Additionally, the striatum is an area heavily enriched in mu-opioid receptors (MORs), which are major targets of the “reward pathway” in that area (Gerdeman et al., 2003;Grueter et al., 2012) and a potential reason for such strong

interactions between HIV-1 infection and opioid drug abuse. Because viral load is very tightly controlled with current cART regimens, but the neurocognitive impairments remain (Heaton et al., 2010; Neuenburg et al., 2002), suggests that the CNS pathology may be very different from HIV-1's effects in the periphery and that a chronic low-level of infection still remains in the CNS that is contributing to these effects (Kopnisky et al., 2007; Johnson et al., 2013). Because the majority of the current cART therapy targets the viral process, but not inflammation, new therapeutics may be necessary to curb the long-term inflammatory processes. A relatively new cART agent, maraviroc, is a CCR5 antagonist with good CNS penetration (Pfizer Inc., 2007). We believe that this agent may have a broad utility in preventing both HIV-1 infection and inhibiting inflammation in the CNS for reasons described in the sections below.

## **HIV-1 virology**

The HIV-1 genome is packaged in a single strand of RNA containing nine genes that are separated into three classes: viral structural proteins, regulatory elements, and accessory proteins (Gallo et al., 1988). The structural proteins are composed of *gag*, *pol*, and *env*, the regulatory elements by *tat* and *rev*, and the accessory proteins by *nef*, *vpr*, *vif*, and *vpu* (Figure 1.2). While several of the translated proteins and protein products have been shown to be neurotoxic, including gp120, gp41, Tat, Rev, Vpr, and Nef (Nath, 2002), the focus of the work in this dissertation will be on two viral proteins, gp120 and Tat, which are the most well-studied in the field.

Viral infection and replication are multi-step processes required for propagation in the host. The viral envelope glycoproteins (gp120 and gp41, collectively known as

gp160) bind to their primary receptor, CD4, as well as a co-receptor, most notably C-C chemokine receptor 5 (CCR5) and C-X-C chemokine receptor 4 (CXCR4), though an array of other chemokine receptors have been shown to be able to act as co-receptors, including CCR2, CCR3, and CX<sub>3</sub>CR<sub>1</sub> (He et al., 1997;Garin et al., 2003;Puissant et al., 2003). A conformational change in gp120 following CD4 binding exposes the binding site of gp120 to allow subsequent co-receptor binding. An additional conformational change in ligand exposes gp41, which is able to penetrate the host cell membrane (Shaw and Hunter, 2012). In brief, following infection, HIV-1 first undergoes reverse transcription of its viral genome in the host cytoplasm to create a cDNA sequence, and then is able to integrate its genome into the host nucleus, enabling the virus to hijack the host's transcriptional machinery for its own uses (Van and Debyser, 2005;Brown et al., 1987).

HIV-1 Tat is a transcriptional transactivator, which is not present in the virus itself, but is synthesized early on in infection. It contains five functional domains in its protein sequence: N-terminal, cysteine-rich, core, basic, and C-terminal. Domains 1-4 are encoded in the first exon, amino acids 1-72, while Domain 5 is encoded in exon 2, amino acids 73-101 (Jeang, 1996;Nath et al., 1996). The length of the Tat protein can vary from 72-101 amino acids depending on the viral clade and splicing. It acts by helping to recruit proteins to the enhancer and promoter elements of the LTR region of the provirus to lift the transcriptional suppression from the promoter region (Rohr et al., 2003). Additionally, Tat can be secreted from the virus (Rayne et al., 2010) and interact with the host extracellularly by binding to membrane receptors, and additionally it can pass through membranes by endocytosis and has a nuclear localization sequence



(Debaisieux et al., 2012). The ability of Tat to interact with the host in a number of ways in addition to its “sticky” basic region, makes HIV-1 Tat a promiscuous and toxic protein.

### **Opioids Exacerbate HIV-1 Infection: the role of glia**

Opioid abuse and HIV-1 infection are closely linked epidemics with about 1/3 of HIV-1 infected people abusing opioids (UNAIDS, 2010). Opioids are involved in both the spread and the pathogenesis of the virus; HIV-1 is often spread through needle sharing, risky sexual behavior, and exchange of sex for drugs. In addition, drug use worsens the pathology of the disease, with opioid abusers 4-times as likely to show HIV-1 encephalitis (HIVE) compared to non-users (Bell et al., 2002). Use of opioid drugs also hastens disease progression (Kumar et al., 2006; Noel, Jr. and Kumar, 2006). In cell culture models opioids are able to potentiate neuronal cell death (Hu et al., 2005; Hauser et al., 2006; Zou et al., 2011b; Gurwell et al., 2001) and it has become clear that the presence of glia are critical to this interaction. Neurons are not infected by HIV-1 (Guha et al., 2012), and astrocytes latently (Messam and Major, 2000) are infected, while microglia support the majority of the active viral replication in the CNS (Rock et al., 2004). Opioid-HIV-1 interactions are limited in the absence of glia (Zou et al., 2011b; Gurwell et al., 2001) and glia have been shown to have synergistically elevated intracellular calcium influx and chemokine secretion in response to morphine and HIV-1 Tat (El-Hage et al., 2008b; El-Hage et al., 2005).

### **The Chemokine System**

Chemokines are a subgroup of chemotactic cytokines, with approximately 50 known to date and along with their 19 receptors, comprise a numerous and diverse group of proteins. The chemokines and receptors are divided into four families based on the location of conserved cysteine residues within the protein sequence, CXC, CC, C, and CX<sub>3</sub>C and are designated as alpha, beta, gamma, and delta chemokines/chemokine receptors, respectively (Allen et al., 2007) with the receptors containing an “R” and ligands containing an “L” in the designation. Chemokines and their receptors do not interact specifically, and the redundancy of the chemokine system at the level of both the ligand and the receptor is important to maintaining proper immune function (Mantovani, 1999;Devalaraja and Richmond, 1999). The ligands are small, 8-12 kDa proteins which bind to their G-protein-coupled receptor counterparts, the majority of which are G<sub>i/o</sub>-coupled, as pertussis toxin (PTX) is able to reverse the majority of the effects (Cartier et al., 2005).

Chemokines are generally categorized into inflammatory and homeostatic, though more and more evidence is beginning to suggest physiological roles for the chemokines. However, the levels of the chemokines are tightly regulated under physiological conditions, though SDF-1/CXCL12 and its receptor CXCR4 as well as fractalkine/CX<sub>3</sub>CL<sub>1</sub> and CX<sub>3</sub>CR<sub>1</sub> are released in higher quantities under basal conditions. These chemokine-chemokine receptor pairs, as well as others, play roles in brain development and neuronal migration, cell proliferation and trophic support, modulation of synaptic transmission, and brain inflammation and neurodegenerative diseases (Bajetto et al., 2001;Cartier et al., 2005).

## Opioid-Chemokine Interactions

The ability for opioid-chemokine interactions occurs on a number of levels. Both opioid and chemokine receptors are predominantly  $G_{i/o}$ -coupled GPCRs and thus may activate some of the same signaling pathways within a cell, though the agonist-specific effects can also be noted. Heterologous desensitization occurs between opioids and chemokines, meaning that activation of one receptor by its ligand prevents or dampens a subsequent stimulation of the other receptor by its ligand, and suggests a functional linkage. Addition of morphine to astrocyte cell lines as well as primary human astrocytes decreases MIP-1 $\beta$  gene expression and secretion with concomitant elevations in CCR5 mRNA and protein (Mahajan et al., 2005a; Mahajan et al., 2005b; Mahajan et al., 2002). Elevations in CCR5 gene expression and decreased  $\beta$ -chemokine production were noted in microglia and cells of peripheral monocyte lineage when exposed to morphine, which has been linked to morphine's enhancements in HIV-1 viral replication in these cells (Guo et al., 2002; Li et al., 2003; Peterson et al., 1990; Steele et al., 2003). Additionally, opioid and chemokine receptors putatively form heterodimers, which have the potential to not only influence the ability of the other receptor to signal, but the agonist-selectivity as well. Studies into receptor interactions have shown evidence for oligomerization of the mu-opioid receptor and CCR5 (Chen et al., 2004; Suzuki et al., 2002). Additionally, heterologous desensitization between MOR and CCR5 has been reported, as MOR agonists can prevent ligand-mediated chemotaxis of immune cells (Chen et al., 2004; Szabo et al., 2002; Szabo et al., 2003), and CCR5 agonists attenuate morphine-induced antinociception (Chen et al., 2007; Szabo et al., 2002).

## **HIV-1 Protein-Chemokine Interactions**

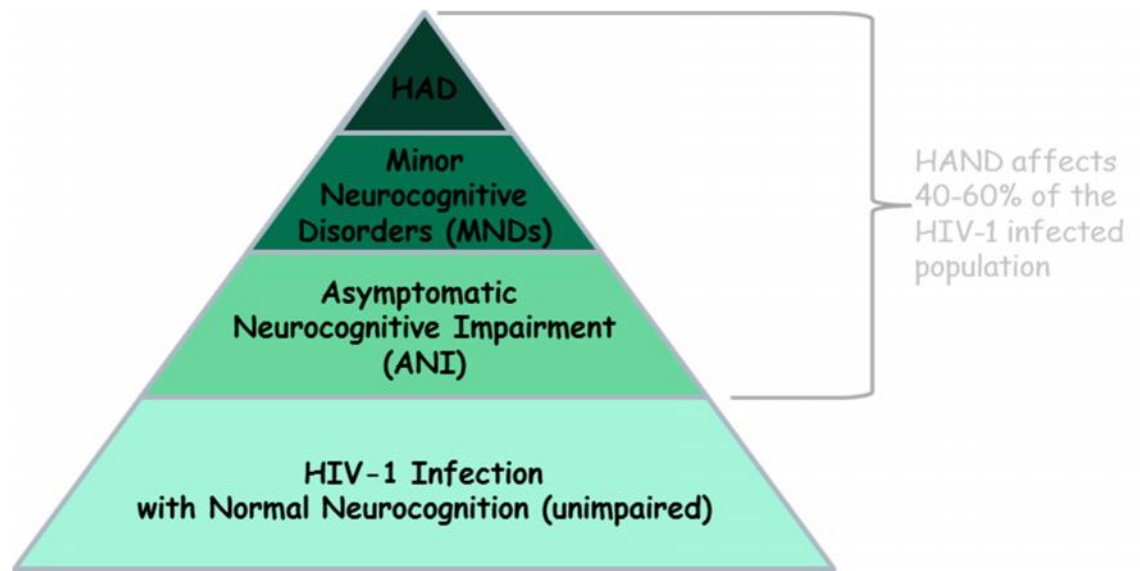
While chemokines and their receptors can modify a number of disease states, their relationship with HIV-1 is different in that chemokine receptors are not only involved in promoting an inflammatory state, but they are also a means of transmitting viral infection. The canonical HIV-1-chemokine relationship is through HIV-1 gp120 molecules and as CCR5 is the major focus of this body of work, we will discuss the interactions through this co-receptor more specifically. Gp120 interactions with CCR5 specifically drive initial infection with HIV-1 (Levy, 2009; Moore et al., 2004). A genetic mutation exists in which there is a 32 base pair deletion in the CCR5 gene ( $\Delta 32$  CCR5), and while individuals heterozygous for the mutation can become infected, but generally exhibit a less severe pathology and generally don't progress to AIDS, those who are homozygous for the mutation are highly resistant to infection (Boven et al., 1999).

These interactions are also specific to cells of the monocyte/macrophage lineage. M-tropic or R5-tropic strains of virus interact with CCR5, while T-tropic (T-lymphocyte-utilizing) strains interact with CXCR4. Because the M-tropic virus is around initially, and because the CNS is infected within the first two-weeks of infection, and the surveying cells in the CNS are macrophage/microglial in nature, the CNS disease state tends to be R5-utilizing. Binding of gp120 to CCR5 is not only able to lead to viral infection, but interactions between gp120 and CCR5, irrespective of CD4 involvement can lead to the activation of cell signaling mechanisms and it appears that these signature events are different in nature than activation of a chemokine receptor by its endogenous ligands (Khan et al., 2004; Kaul et al., 2007). It has been reported that

signaling by chemokine ligands, while producing inflammatory effects, also produced pro-survival signals, while stimulation by gp120 produced pro-apoptotic events.

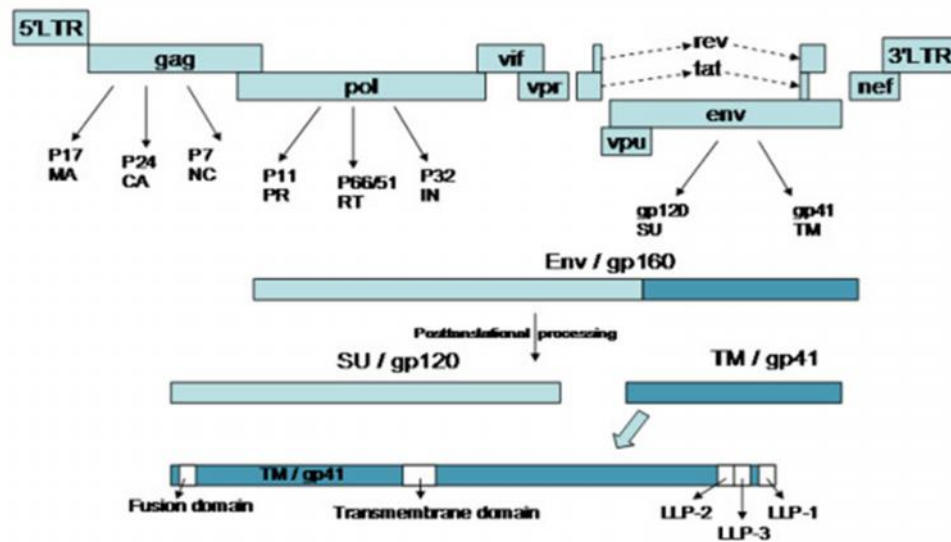
HIV-1 Tat, has a number of binding partners and not much work has been performed to suggest that CCR5 is a strong target, however HIV-1 Tat does share some sequence homology with the  $\beta$ -chemokines (de et al., 2000; Albini et al., 1998), containing a di-cysteine sequence among other conserved amino acids, and has been shown to cause chemotaxis of immune cells in a  $\beta$ -chemokine receptor-dependent manner. Additionally, our lab has provided evidence that the upstream signaling events produced by Tat's interactions with glia may converge on CCL5/CCR5 (El-Hage et al., 2008b). Tat treatment significantly upregulates RANTES production, and knockout of CCL5 prevents morphine and Tat-induced gliosis in mice (El-Hage et al., 2008b; El-Hage et al., 2008a). The numerous interactions of the opioid system with chemokines as well as its relationship to HIV-1 make the chemokine system, and CCR5 in particular a good target to examine the interactive effects of opioid abuse and infection.

**Figure 1.1**



**Figure 1.1** Classifications of HIV-1 associated neurocognitive disorders (HAND). HAND affects approximately 40-60% of the infected population and to a wide range of degrees. About half of the population is considered normal (unimpaired), while the rest of the population can experience minor deficits, termed asymptomatic neurocognitive impairment (ANI), more moderate symptoms, minor neurocognitive disorders (MNDs), or the most severe form called HIV-1 associated dementia (HAD). Figure adapted from (Ellis et al., 2007) .

**Figure 1.2**



**Figure 1.2** The HIV-1 genome is composed of nine genes (*gag*, *pol*, *vif*, *vpr*, *rev*, *tat*, *env*, *vpu*, and *nef*) which are flanked on either side by long terminal repeat regions. The Env protein (gp160) is further processed into gp120 and gp41 protein products. Image taken from (Costin, 2007).

## Chapter 2

### **CCR5-Utilizing gp120<sub>ADA</sub> has Modest Effects on Striatal Neural Cells**

#### **Alone and Does not Show Interactions with Morphine.**

*(This chapter, in part, was published as a paper in the Journal of Neuroimmune Pharmacology in December of 2012, 7(4):877-91, entitled Morphine and gp120 toxic interactions in striatal neurons are dependent on HIV-1 strain)*

#### **Introduction**

While it is generally accepted that neurons do not have the machinery for viral infection (An et al., 1999;Takahashi et al., 1996;Wiley et al., 1999), they are still subject to direct actions of virus or of the secreted envelope viral protein, gp120, among others, leading to cell death, injury, and aberrant signaling (Meucci et al., 1998;Khan et al., 2004;Singh et al., 2004;Kaul et al., 2007). In addition to direct effects, neuronal injury is likely produced in response to gp120 by indirect actions through activation of microglia cells (Dreyer et al., 1990;Lipton et al., 1991;Kaul et al., 2007), and alteration of astroglial function (Pulliam et al., 1993;Benos et al., 1994b;Benos et al., 1994a;Holden et al., 1999).

While multiple strains of gp120 exist, which are designated based on their receptor interactions, R5-virus, which is thus named for its preference for binding to the CCR5 receptor is the variant that is predominantly responsible for CNS infection (Schnell et al., 2011;Gonzalez-Perez et al., 2012). Accordingly, this is of particular interest in determining the role of specific viral proteins in the neuropathological



changes that occur with HIV-1 infection. Opioid abuse in those infected with HIV-1 is a co-morbid factor augmenting the CNS consequences of the disease (Bell et al., 1998; Bell et al., 2006; Nath et al., 2000; Nath et al., 2002; Gurwell et al., 2001; Kumar et al., 2004; Hauser et al., 2005; Turchan-Cholewo et al., 2006; Banerjee et al., 2011). While multiple proteins are likely involved in opioid exacerbation of the CNS pathology of HIV-1, opioids have been shown to specifically interact with R5-tropic gp120 and/or the  $\beta$ -chemokine system. Morphine elevates CCR5 receptor and its chemokine ligands' gene expression in human astrocytes, with interactive effects with gp120 found in an astroglial cell line (Mahajan et al., 2005a; Mahajan et al., 2005b; Mahajan et al., 2002). Additionally gp120 can also modify expression levels of the opioid system (Turchan-Cholewo et al., 2008; Fitting et al., 2010b). In addition to viral protein-opioid interactions, the endogenous chemokine ligands of CCR5 also modulate the opioid system which is thought to be caused by either hetero-oligomerization of the opioid and chemokine receptors (Chen et al., 2004; Suzuki et al., 2002) or through heterologous desensitization based on similar signaling mechanisms through  $G_{i/o}$ - or  $G_q$ -coupling (Mueller et al., 2002; Kraft et al., 2001; Law and Loh, 1999; Law et al., 2000). This has been shown both in CCR5 agonist-mediated blockade of morphine antinociceptive activity (Szabo et al., 2002; Chen et al., 2007), and mu-opioid-induced inhibition of immune cell chemotaxis (Chen et al., 2004; Szabo et al., 2003; Szabo et al., 2002). And not only do gp120 or the chemokines interact with opioids to modulate their function, but the presence of  $\beta$ -chemokines with R5-gp120 has been shown to block gp120-mediated neurotoxicity (Kaul et al., 2007; Avdoshina et al., 2010), and thus provides another level of complexity to interactions between these two systems.

As both neurons and glia express CCR5 as well as MOR, it is challenging to determine the role of each cell type in the interactive pathogenesis of HIV-1 in opioid abusers (Peterson et al., 1990; Peterson et al., 1998; Chao et al., 1994; El-Hage et al., 2005; Hu et al., 2005; Turchan-Cholewo et al., 2006; Zou et al., 2011a). Previous studies examining HIV-1 Tat interactions with morphine have uncovered a role for both neurons and glia in the response, with a specific role for glia in the opioid component (Zou et al., 2011b). While Tat was able to produce synergistic neurotoxicity when combined with opioids, it was unclear whether this interaction would extend to other HIV-1 proteins. A previous study in our lab has already examined gp120 toxicity from an X4-strain in neuron cells alone and in the absence of opioid co-application (Singh et al., 2004). Thus we undertook to expand our studies with gp120 to a more neurotropic strain, and to examine the interactions of this particular type of gp120 molecule with morphine to see if it could modulate gp120's neurotoxic response in our hands.

## **Materials and Methods**

Experimental protocols conformed to local Institutional Animal Care and Use Committee (IACUC) and national Policy on Human Care and Use (PHS) guidelines on the care and ethical use of animals. Experiments were designed to minimize the total number of animals used and their discomfort.

### **Primary Mixed Glia Cultures**

Mixed glial cultures, containing astroglia and microglia at ~92% and 8%, respectively (Zou et al., 2011b) were prepared from the striata of post-natal day 0 - 1

(P0 - P1) Imprinting Control Region (CD1) mouse pups (ICR; Charles River Laboratories, Boston, MA). Six to 8 pups were decapitated, brains removed from skulls and striata dissected away from the cortex. Tissue was minced and incubated in trypsin (2.5 mg/mL; Sigma-Aldrich Co., St. Louis, MO) and DNase (0.015 mg/mL; Sigma-Aldrich Co.) in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen Corp., Carlsbad, CA) containing glucose (27 mM; Sigma-Aldrich Co.),  $\text{Na}_2\text{HCO}_3$  (6 mM; Invitrogen), and penicillin / streptomycin (100 U/mL and 100  $\mu\text{g}/\text{mL}$ ; Invitrogen) for 30 min. at 37°C and 5%  $\text{CO}_2$  / 95% air with intermediate mixing. Tissue was centrifuged at 1000 x g for 5 -10 min, resuspended in 10 mL of glial maintenance media containing DMEM with the above supplements along with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT). Tissue was mechanically triturated using 10 mL and 5 mL pipettes sequentially, and which possess decreasing bores, then filtered through 100  $\mu\text{m}$  nylon mesh pores (BD Falcon; Bedford, MA). Tissue was centrifuged at 1000 x g as performed above, resuspended in 3 mL of glial maintenance medium, then further triturated using a 5 mL pipette, filtered through a 40  $\mu\text{m}$  cell strainer and plated at  $1.5 - 2.0 \times 10^5$  cells/ $\text{cm}^2$  on poly-L-lysine coated cell culture dishes. Media was exchanged ~24 h after plating, day 1, and every 3 d following with glial maintenance media until confluence was reached (~10-14 d). Cells were treated in supplemented DMEM lacking FBS.

### **Primary Neuronal Cultures**

Neuronal cultures were prepared from the striata of embryonic day 15 – 16 (E15 - 16) ICR mice. Dams were anesthetized with isoflurane and following cervical dislocation, embryos were aseptically removed from the uterus of the timed pregnant animal. Brains from 4 – 6 pups were removed and striata were dissected away from the cortex. Tissue was minced and incubated in trypsin and DNase in neurobasal media (NBM; Invitrogen) for 30 min at 37°C and 5% CO<sub>2</sub> / 95% air using intermittent inversion. Digested tissue was centrifuged at 1000 × g for 5 - 10 min and resuspended in NBM containing B27 (1x; Invitrogen), L-glutamine (0.5 mM; Invitrogen), glutamate (0.025 mM; Sigma-Aldrich Co.), and penicillin/streptomycin (100 U/mL and 100 µg/mL, respectively). Tissue was mechanically digested using a 1 mL pipette and filtered through a 70 µm cell strainer (BD Falcon). For co-cultures, cells were plated at  $2.5 \times 10^4$  cells/cm<sup>2</sup> onto 10 day in vitro (DIV) mixed glia and maintained in supplemented NBM (Figure 2.1). When neurons were cultured alone they were plated, at the same density as above, onto poly-L-lysine-coated cell culture dishes and maintained as described above. Media was exchanged 24 h after the culture and every 2 days thereafter until experimentation at DIV 8-10. Cells were treated in supplemented NBM without the addition of glutamate.

### **MOR and CCR5 Immunofluorescence**

Striatal neurons grown in culture for 9-10 days and striatal glial cells grown for 10-12 days on poly-L-lysine coated coverslips and remaining in the untreated state were rinsed 2x in 4°C PBS and fixed for 15 min in 4% paraformaldehyde. Coverslips were then rinsed 3 × 10 min in PBS, permeabilized in PBS containing 0.1% Triton-X 100

(Shelton Scientific Inc., Shelton, CT) and 0.1% BSA (Sigma-Aldrich Co.) for 15 min, and subsequently blocked supplemented with 0.1% BSA and 1% goat serum (Invitrogen) in PBS for 1 h at room temperature. Dual-immunofluorescent labeling was performed by incubating cells overnight at 4°C in rabbit anti-MOR antibodies (1:2500, Antibodies Incorporated, Davis, CA, 61-204) or mouse antiserum to CCR5 (1:100; BD Pharmingen, San Diego, CA; 555991) and co-localized with either microtubule associated protein 2 (MAP2; mouse anti-MAP2, MAB378, Millipore, 1:000, or rabbit anti-MAP2, Abcam, ab32454 1:000) or glial fibrillary acidic protein (GFAP; mouse anti-GFAP, Millipore, MAB360, 1:750, or rabbit anti-GFAP, AB5804, 1:750). Cells were rinsed 3 × 10 min in PBS and secondary antibodies against the primary host, which included goat anti-rabbit Alexa-488, donkey anti-mouse Alexa-594, goat-anti-rabbit Alexa 594 and goat anti-mouse Alexa-488 (all from Invitrogen), were applied for 1 h at room temperature, followed by counterstaining with Hoechst 33342 (0.5 µg/mL; Invitrogen) to identify cell nuclei. Coverslips were mounted on slides with Prolong Gold Antifade Reagent (Invitrogen) and dried overnight in the dark. Fluorescence was visualized and digital images acquired using a Zeiss LSM 700 confocal module configured to an Axio Observer Z.1 microscope and Zen 2010 software (Zeiss Inc., Thornwood, NY).

### **Time-lapse Microscopy**

Eight fields of neurons were selected (40x magnification, MosaiX module, Zeiss AxioVision 4.6) to repeatedly track using a computerized microscope stage encoder and tracking software (Zeiss, AxioVision 4.6; Mark&Find, MosaiX and Time Lapse modules).

Time-lapse digital images of cells were recorded at 30 min intervals for 72 h using a microscope and automated, computer-controlled stage encoder with environmental control (37°C, 95% humidity, 5% CO<sub>2</sub>) that allowed us to track the same neurons over time (Axio Observer Z.1, Zeiss). Preselected neurons were assessed for viability using rigorous morphological criteria, including neurite disintegration, loss of phase brightness, and either involution or complete fragmentation of the cell body (Singh et al., 2005; Zou et al., 2011a; Bakalkin et al., 2010). Our methodologies have been previously confirmed by staining cells with ethidium monoazide (Buch et al., 2007), which identifies dead cells. Neuron viability was assessed by tracking the cells at each time point over the 72 h experimental period to determine whether they lived or at what time they died. Two-way repeated measures ANOVA was performed to assess the effect of treatment at 4 h intervals on neuronal survival which was expressed as a percent of the pretreatment value. Assessments of neuron viability were made separately in neurons cultured alone or plated on top of a layer of mixed glia. Cultures were treated with glutamate-free neurobasal medium, but which contained the other supplements, either alone or in combination with morphine sulfate (500 nM; NIDA Drug Supply System); gp120<sub>ADA</sub> (500 pM; Immunodiagnostics Inc., Woburn, MA), or morphine + gp120<sub>ADA</sub>.

### **Mixed glial reactive species production**

Reactive oxygen species were measured using the cell-permeable dye, 2',7'-dichlorofluorescein diacetate (DCF-DA), which when inside the cell is hydrolyzed by cytosolic esterases to its non-cell-permeable form (DCF) and which fluoresces when in contact with the ROS/RNS species H<sub>2</sub>O<sub>2</sub>, HO<sup>•</sup>, ROO<sup>•</sup>, <sup>•</sup>NO, and ONOO<sup>-</sup> (Crow,

1997;Wang and Joseph, 1999;Gomes et al., 2005). Measurements were performed on a PHERAstar plate reader (BMG Labtech, Cary, NC); the plates were read at  $\lambda_{\text{ex}} = 488$  nm and  $\lambda_{\text{em}} = 525$  after 90 min of treatment with concentrations of gp120<sub>ADA</sub> ranging from 0.1 pM to 10 nM.

### **ERK1/2 phosphorylation**

Activation of ERK1/2, measured through changes in the level of the phosphorylated species was assessed through an AlphaScreen SureFire p-ERK1/2 cellular kinase assay (PerkinElmer, Waltham, MA). Confluent mixed glia grown on 96-well plates for 10-14 d in vitro were serum starved overnight in our normal maintenance medium lacking FBS (SFM). Before treatment, media was exchanged for fresh SFM and Morphine (500 nM), DAMGO (500 nM), RANTES (100 ng/mL), and gp120<sub>ADA</sub> (500 pM) were added to cells for 1.0, 5.0, 7.0, 15, 30, and 60 min. Following each time-point, medium was removed, cells were rinsed in cold PBS (4°C) and 100  $\mu$ L of lysis buffer was added to the well and incubated for 10 min with shaking. To a 384-well opaque, white ProxiPlate (PerkinElmer), 4  $\mu$ L of each cell lysate was added in addition to positive and negative control lysates. To each treatment lysate, 7  $\mu$ L of a reaction mix containing streptavidin-coated donor beads and protein A-coated acceptor beads linked to antibodies against ERK and the phosphorylation site of ERK (Thr202 or Tyr204), respectively. Plates were sealed, wrapped in foil and mixed on an orbital shaker for 1-2 min, then incubated for 2 h at room temperature. Plates were read on a PheraStar microplate reader (PerkinElmer) with excitation at 680 nm and emission at 520-620 nm.

Levels of p-ERK1/2 were subtracted from the blank value and converted to fold-increase over the control value.

### **Fluorescence assessment of NF- $\kappa$ B p65 nuclear translocation**

Mixed glial cultures grown for 10-12 days on coverslips were treated for 12 h with serum free media alone, or containing morphine sulfate (500 nM; NIDA Drug Supply System), gp120<sub>ADA</sub> (500 pM; Immunodiagnostics Inc., Woburn, MA), or morphine + gp120<sub>ADA</sub>. Following treatment, cells were rinsed 2x in 4°C PBS and fixed in 4% paraformaldehyde for 15 min. Fixed cells were rinsed 3 × 10 min in PBS, permeabilized in PBS containing Triton X-100 (Shelton Scientific Inc.) and blocked in PBS supplemented with 0.1% BSA and 1.0% goat serum, both for 15 min. Monoclonal antiserum created in mouse against NF- $\kappa$ B p65 (Santa Cruz Biotechnology; Santa Cruz, CA, sc-8008, 1:100) as well as rabbit anti-glial fibrillary acidic protein (anti-GFAP; Chemicon, AB5804, 1:750) were incubated on cells overnight at 4°C. Cell nuclei were counterstained with Hoechst 33342 (0.5 µg/mL; Invitrogen, Molecular Probes).

## **Results**

### **MOR and chemokine receptor immunoreactivity on neurons and glia**

MOR, CXCR4, and CCR5 immunofluorescence was colocalized in untreated striatal neurons and astrocytes by confocal microscopy to assess the extent to which the principal molecular targets of opioid drugs and gp120 were present and to assess their cellular distribution in each cell type. Receptors were co-localized in neurons



(Figure 2.2 **a-c**) or astrocytes (Figure 2.2 **d-f**), respectively, using antibodies against MAP2 or GFAP; cells were counterstained with Hoechst 33342. In neurons, CXCR4, CCR5, and MOR immunoreactivity was associated with the cell body and dendrites, while CCR5 immunofluorescence often continued into the axolemma (**a-c**). The proportion of CXCR4 and CCR5 immunoreactive neurons was  $69.3 \pm 0.7\%$  and  $77.4 \pm 1.7\%$ , respectively, which also infers that a subset of neurons co-express both chemokine receptors. Astrocytes tended to display punctate patterns of CXCR4, CCR5, and MOR immunolabeling (**d-f**); faint, diffuse receptor reactivity was occasionally evident at the cell membrane (e.g., CCR5 in **e**). The proportion of CXCR4 and CCR5 immunofluorescent astrocytes was  $45.2 \pm 1.6\%$  and  $43.8 \pm 2.4\%$ , respectively.

### **Neurotoxicity induced by gp120<sub>ADA</sub> and morphine**

Analyzing the response of individual neurons repeatedly throughout the experiment eliminates inter-subject variability and permits subtle treatment effects to be revealed through a repeated measures ANOVA (Singh et al., 2004; Singh et al., 2005; Bakalkin et al., 2010; Zou et al., 2011b). The use of computer-aided, near-continuous tracking of large numbers of neurons (and glia) greatly facilitates this task, while increasing the sensitivity of the assay. This strategy has been used by us to examine neuroprotective effects of silencing the phosphatase and tensin homolog on chromosome 10 (PTEN) on HIV-1 gp120<sub>IIIB</sub>-induced neurotoxicity (Zou et al., 2011a), to demonstrate the role of glia in morphine-induced exacerbation of Tat neurotoxicity (Zou et al., 2011b), and to show that mutations in dynorphin A underlie spinocerebellar ataxia type 23 (Bakalkin et al., 2010). In the present work, tracking the responses of individual

neurons has revealed sometimes subtle differences in the cytotoxic effects of gp120. To test whether opiate-gp120 interaction occurred in an R5-tropic strain of gp120, gp120<sub>ADA</sub> was tested through time-lapse microscopy in our co-culture paradigm. Gp120<sub>ADA</sub> alone was toxic to neurons in the presence of glia, but only displaying significant increase in the rate of neuron death (time × treatment) compared to controls rather than mean differences at any particular time (Figure 2.3). Additionally, morphine failed to enhance gp120<sub>ADA</sub>-induced cell death. Repeated measures ANOVA, data are the mean ± SEM of n = 4 experiments.

### **Effects of morphine and gp120 on astroglial ROS**

The effects of gp120<sub>ADA</sub> on ROS production has not been systematically explored. Previous studies have been conducted on the effects of morphine on ROS production and weren't repeated (Zou et al., 2011b). Gp120<sub>ADA</sub> produced upregulations in astroglial ROS with an EC<sub>50</sub> =  $0.54 \times 10^{-9}$  nM and significant elevations from control at  $1 \times 10^{-9}$  and  $1 \times 10^{-8}$  M (\* P < 0.05 vs. control; Figure 2.4). One-way ANOVA with Dunnett's post-hoc test; data are the mean ± SEM of n = 3 experiments.

### **Alphascreen Surefire p-ERK1/2 assay**

Time courses of activation of p-ERK1/2 by MOR and CCR5 agonists showed ligand-specific effects (Figure 2.5). Morphine treatment between 5.0 and 7.0 minutes showed an upward trend in the level of p-ERK followed by a decrease in the activated transcription factor at 30 and 60 min, with a significant decrease at 60 min (\* P < 0.05 vs. control) (**A**). DAMGO, a selective MOR agonist showed a similar shape in the time-

course, but showed no significant differences in p-ERK levels over time (**B**). An endogenous CCR5 agonist, RANTES, showed increases of p-ERK over time with a significant increase at the 60 min time-point (\*  $P < 0.05$  vs. control) (**C**). Conversely, the exogenous CCR5 agonist, gp120<sub>ADA</sub> showed no effect on p-ERK activation (**D**). Data are the mean  $\pm$  SEM of  $n = 4$  experiments, and were analyzed using ANOVA with Tukey's post-hoc analysis.

### **NF- $\kappa$ B Activation is Viral Protein-Selective**

The ability of gp120<sub>ADA</sub> to activate a pathway known to be involved in Tat-mediated inflammatory effects (El-Hage et al., 2008b) was examined by assessing the nuclear translocation of NF- $\kappa$ B p65 via immunofluorescent labeling of astroglia (Figure 2.6. Nuclear immunopositivity of GFAP-labeled cells was easily noted, as indicated by cell nuclei with “\*” symbols (**A**). Treatment with gp120<sub>ADA</sub> caused very little nuclear translocation of p65, and was no different from control levels, while Tat produced a robust activation response (\*  $P < 0.05$  vs. control) (**B**). Data are the mean  $\pm$  SEM of  $n = 3$  experiments and were analyzed using a one-way ANOVA with Tukey's post hoc test.

### **Discussion**

Soluble gp120 has been well documented to have effects in both neurons (Meucci et al., 1998;Kaul et al., 2007;Lipton et al., 1991) and in glia (Lipton et al., 1991;Benos et al., 1994b), and the preceding studies attempted to examine a neurovirulent strain of gp120, the R5-tropic gp120<sub>ADA</sub> (Jones and Power, 2006;Ohagen et al., 2003) in our striatal neuron mixed-glial co-culture system to determine if this

strain of gp120 acts in similar fashion to those of other tropisms under study, as well as to see if it interacted with the opioid system. However, while gp120 did show a significant time  $\times$  treatment effect in the time-lapse studies, the effect was not augmented by morphine, though neither was it attenuated. Interestingly, assessment of CXCR4-utilizing gp120<sub>IIIB</sub> (Podhaizer et al., 2012) (Figure 2.7) produced significant neurotoxicity alone and produced a transient increase in cell death when combined with morphine at the 12 h time point, which was highly reproducible, but which did not extend out to the 72 h treatment time. Replicating the above experiments using the CXCR4-CCR5-utilizing (dual-tropic) gp120<sub>MN</sub>, we noted a similar level of neuron death when gp120<sub>MN</sub> was applied alone, and interestingly, also saw a significant and persistent increase in neuron death in combination with morphine (Figure 2.8). It is important to note that the interactive effect with morphine only occurs in the presence of glia (Figure 2.7), while the gp120 toxicity does not require co-culture conditions to produce its effect and is in agreement with our previous findings (Zou et al., 2011a).

Not only did both gp120<sub>IIIB</sub> and gp120<sub>MN</sub> produce significant effects with morphine that were dependent on glia, but their effects of gp120 alone were dependent on effects in the neurons. An initial examination was made into the cell types(s) responsible for the neurotoxic effects of the gp120<sub>ADA</sub> strain by performing time-lapse experiments with morphine and gp120<sub>ADA</sub> in neurons cultured alone. While further study is needed, the preliminary findings suggest that for gp120<sub>ADA</sub> to be neurotoxic, that the presence of glia be required, as neuronal survival in these experiments overlaid that of the control condition. Others have reported that from their observations and experiences that glia, and especially microglia, are required for neurotoxic functions of gp120 to be produced

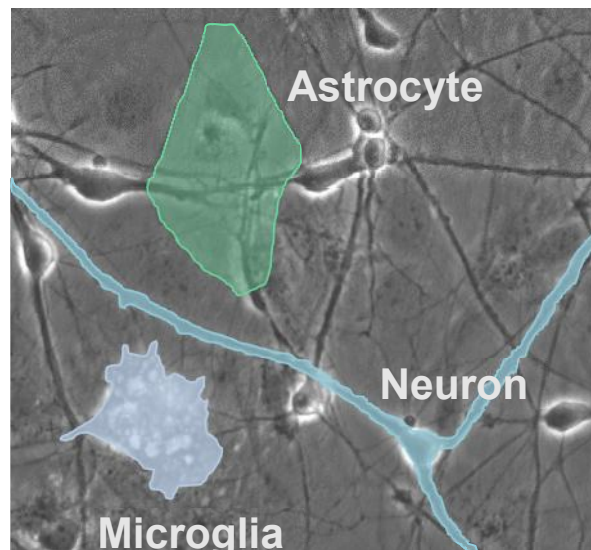
(Kaul et al., 2007;Garden et al., 2004) and cite numerous studies in which microglia treated with either whole virus or soluble protein become activated and release inflammatory signals and or cause neuron damage. However, the strains of virus/gp120 used are either dual-tropic HIV-1<sub>SF2</sub> or R5-tropic HIV-1<sub>SF162</sub> in nature (Koenig et al., 1986;Giulian et al., 1990;Kaul and Lipton, 1999). While their findings in dual-tropic strains are not supported by our data, where little glial activation was seen in measures of ROS, p-ERK1/2 activation, and NF-κB p65 nuclear translocation, and then only at supraphysiological levels, it is intriguing that no X4-strains of virus are reported. It is possible that actions on a specific cell-type are dependent on viral strain. In our hands, gp120<sub>ADA</sub> was unique in its inability to interact with morphine and its somewhat attenuated level of neurotoxicity compared to the other strains of gp120. Studies by others examining a range of gp120 strains showed that levels of toxicities between strains and within a strain were similar, but that the signaling mechanisms through which they achieved the neurotoxic effect were not equivalent (Kaul et al., 2007). It is interesting to note that in our studies, the two strains of virus that were able to interact with morphine also acted through neuronal mechanisms, while gp120<sub>ADA</sub>, which does not interact with morphine seems to require the glia for its effect. It is possible that the integration of signals from multiple sources is required for morphine's augmentation of gp120-mediated effects. While it would be difficult to speculate on the possible root of this effect and the specific signals required, it is known that receptors for gp120<sub>ADA</sub> and morphine have been shown to putatively form heterodimers (Chen et al., 2004;Suzuki et al., 2002), and to interfere with each other's signaling events through heterologous desensitization (Mueller et al., 2002;Kraft et al., 2001;Law and Loh, 1999;Law et al.,

2000). It is possible that activation of both CCR5 and MOR on the same cell could actually prevent further toxicity through desensitization of signaling pathways. For this to have been the cause, it may have been expected, in this scenario, that morphine would have attenuated gp120's toxic effect. However, the time it takes for the receptors to desensitize (morphine takes ~ 16 h), could be enough time for gp120<sub>ADA</sub> to have induced the inflammatory pathway and begun producing downstream inflammatory signaling cascades (Luo et al., 2002; Luo et al., 2003; El-Hage et al., 2008a). CCR5 is an interesting receptor in that it promotes paradoxical responses. While gp120 causes neurotoxicity, it is also able to upregulate neuroprotective signals such as BDNF (Avdoshina et al., 2010) and morphine and an R5-gp120 have been shown to upregulate RANTES, which also affords protection against gp120 itself through competitive binding for the CCR5 receptor (Mahajan et al., 2005a; Kaul et al., 2007). Clinically, higher levels of RANTES in the CSF has been correlated with better neuropathological outcomes, suggesting that RANTES plays an important role, however the opposite effect has been shown as well, so it is still controversial (Kelder et al., 1998; Christo et al., 2009; Letendre et al., 1999). As RANTES is not the only cytokine/chemokine present, it may be difficult to parse out the role of each one, and confounding effects are easily seen so it is difficult to determine whether in that case RANTES is protective or neurotoxic.

Further studies should complete the determination of the cell type(s) involved in gp120<sub>ADA</sub>-mediated toxicity. While it was disappointing that gp120<sub>ADA</sub> produced no interactive responses, it will be interesting to see how the strain of gp120 and the particular chemokine receptor to which it binds can alter the signaling systems. While

there is some evidence for CXCR4-MOR interactions, which could affect opioid-HIV-1 signaling in other cells, the receptors themselves have not been well studied (Toth et al., 2004), nor the downstream signaling pathways if they do exist.

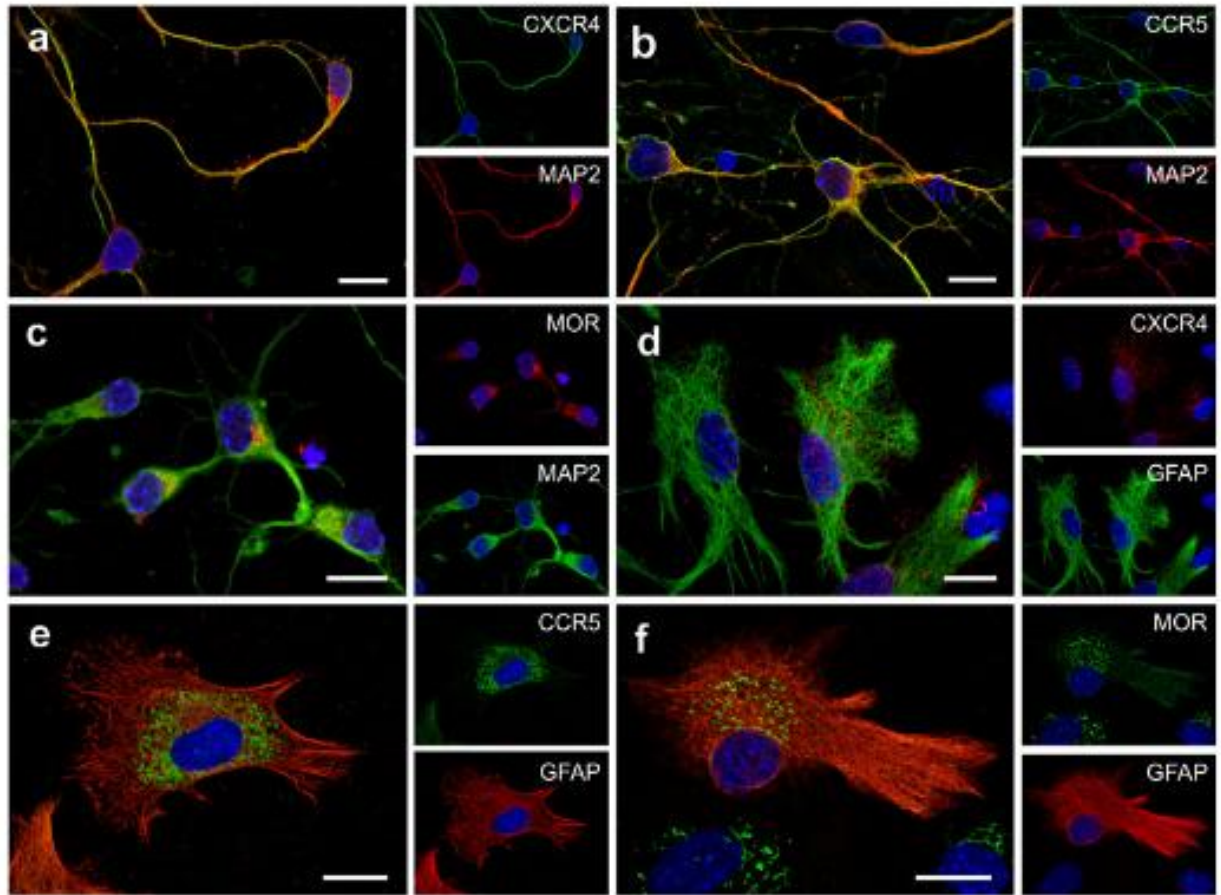
**Figure 2.1**



**Figure 2.1** Representative image of neuron-mixed glia co-culture. Mixed glia from the striatum isolated from P0-2 ICR mouse pups were grown for 10 d until confluent. Isolated striatal neurons were plated on top of the glial bed-layer and the co-culture was allowed to mature for 8 d before treatments were performed. Phase contrast image taken at 40x magnification on an AxioObserver Z.1 microscope (Zeiss Inc) with the major cell types, astrocyte (green), microglia (white) and neuron (blue) indicated.

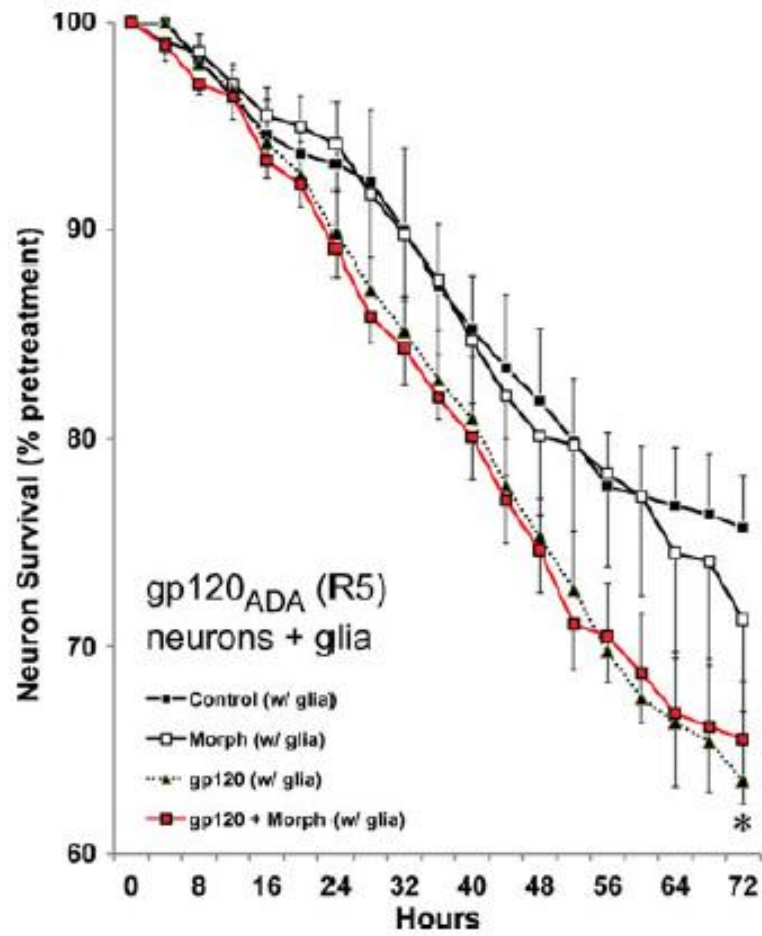
**Figure 2.2**





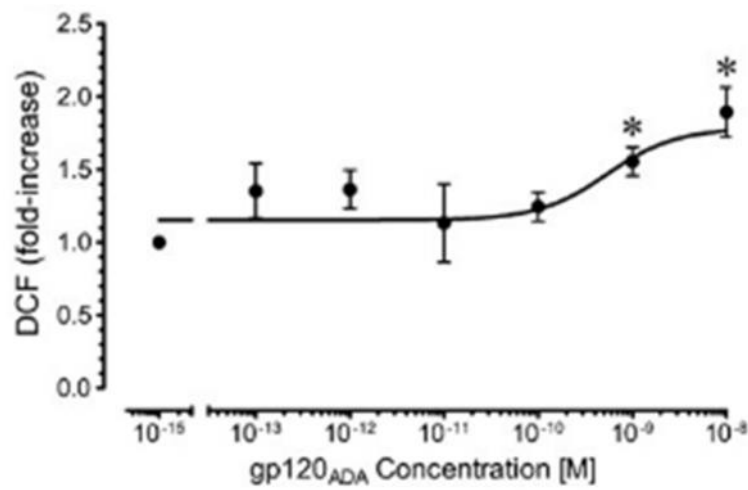
**Figure 2.2** Cellular localization of CXCR4, CCR5, and/or MOR immunofluorescence in striatal neurons and astrocytes. Receptors were colocalized in subsets of neurons (a-c) or astrocytes (d-f), respectively using antibodies against MAP2 or GFAP. Cells were counterstained with Hoechst 33342 (blue). In neurons, CXCR4 (a), CCR5 (b) and MOR (c) immunoreactivity was associated with the cell body and dendrites, while CCR5 antigenicity appeared to extend to some axons (b). In GFAP-immunolabeled astrocytes, CXCR4 (d), CCR5 (e), and MOR (f) displayed a punctate pattern of labeling with some faint reactivity extending into the cell processes (e-f); scale bar = 10  $\mu$ m.

**Figure 2.3**



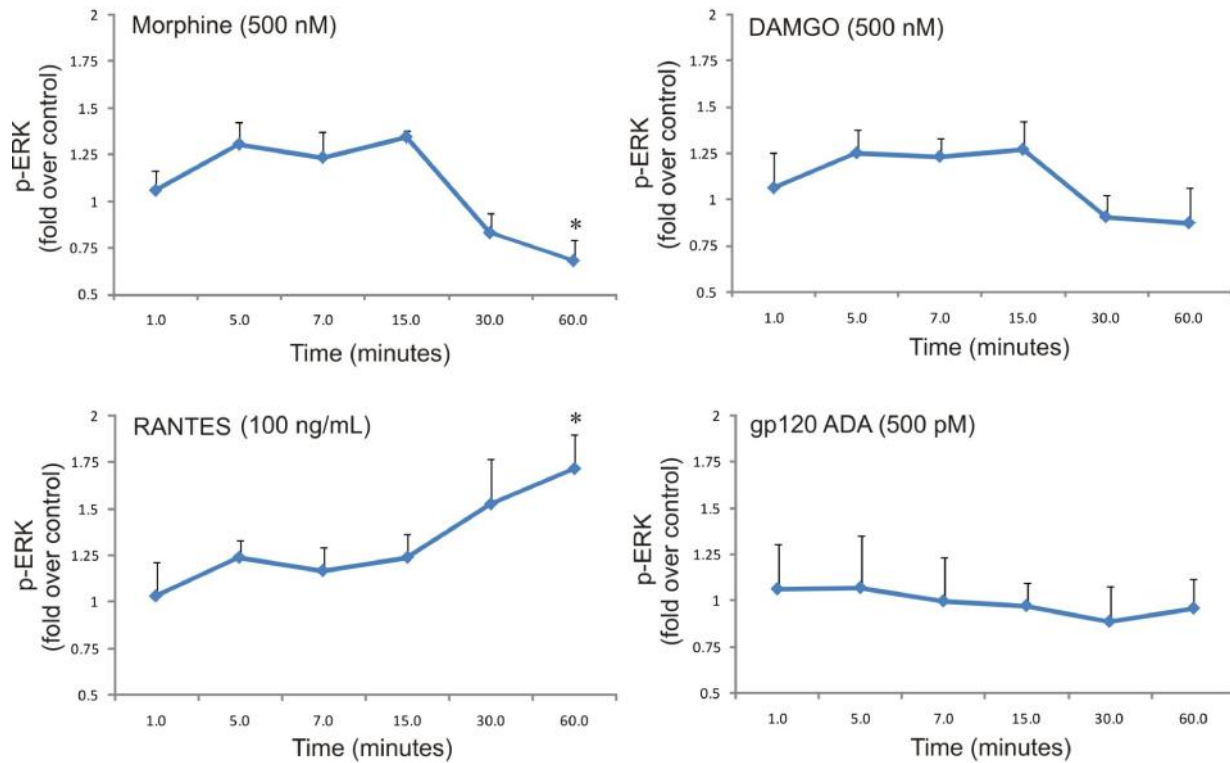
**Figure 2.3** Time-dependent effects of exposure to morphine (Morph) and/or R5-tropic HIV-1 gp120<sub>ADA</sub> in neuron and mixed-glial co-cultures for 72h. Analyses for R5-tropic gp120<sub>ADA</sub> showed a main effect for time, but not for treatment. Although there was a significant interaction effect (time x treatment), Duncan's post-hoc testing did not reveal significance at particular times. When the analysis was simplified by eliminating the morphine group, there was a decline in survival for R5-tropic gp120<sub>ADA</sub> exposed neurons at the last time point of assessment (\* $p < 0.05$  vs. control at 72 h). Otherwise, values for gp120<sub>ADA</sub> treatment alone vs. control (e.g.,  $p = 0.056$  at 68 h) for gp120<sub>ADA</sub> + morphine (e.g.,  $p = 0.054$  at 56 h) were close, but not significant; data are the mean number of surviving neurons  $\pm$  SEM from  $n = 4-6$  experiments.

**Figure 2.4.**



**Figure 2.4** Effects of gp120 on ROS production in mixed-glia assessed by DCF fluorescence. A concentration-effect curve revealed significant increases in DCF relative fluorescence following 45 min exposure to gp120<sub>ADA</sub> with an EC<sub>50</sub> =  $0.54 \times 10^{-9}$  (\* P < 0.05). Data are the mean  $\pm$  SEM of n = 3 experiments.

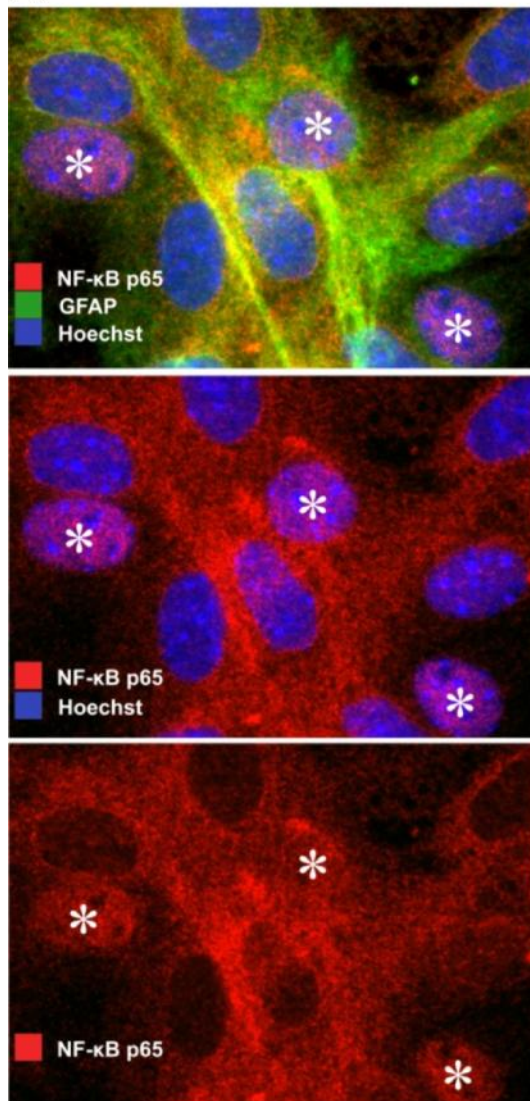
**Figure 2.5**



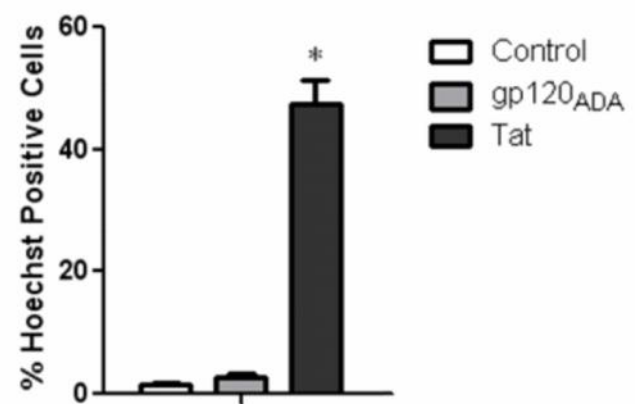
**Figure 2.5** MOR and CCR5 agonists alter ERK phosphorylation in an Alphascreen Surefire assay. A time-course revealed changes in fluorescence suggesting alterations in p-ERK levels during treatment. Morphine showed a decrease in p-ERK levels after 60 min, while levels in response to the selective MOR agonist DAMGO were not significantly modified. The CCR5 agonist, RANTES, upregulated p-ERK after 60 min, while gp120<sub>ADA</sub> had no effect. Data are the mean  $\pm$  SEM of  $n = 3$  experiments.

Figure 2.6

A

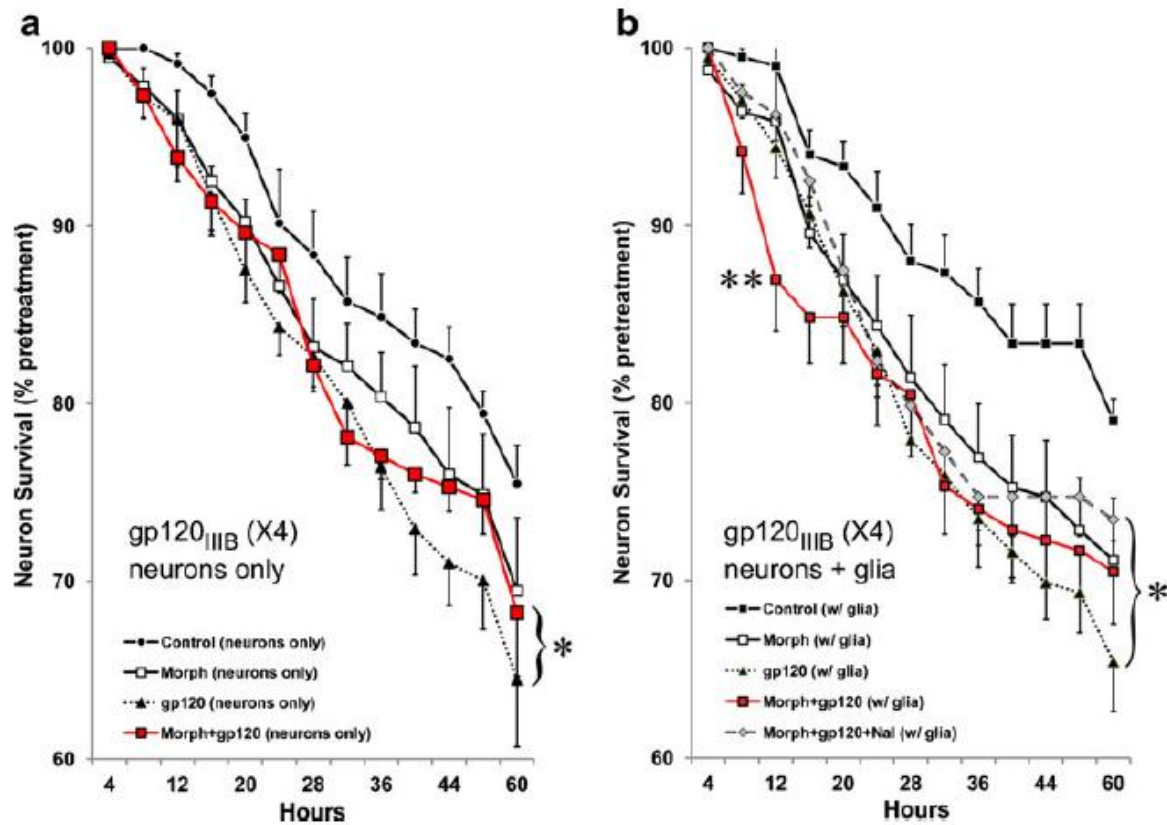


B



**Figure 2.6** Astroglial induction of NF- $\kappa$ B p65 in response to HIV-1 proteins. Mixed-glial cultures were treated for 12 h with media control, 500 pM of gp120<sub>ADA</sub>, or 100 nM of Tat, which was used in this study as a positive control and labeled with p65 antibody. Immunopositivity of the nucleus was easily noted (A) in astroglia; cells in which p65 movement occurred are denoted with a \* over their nuclei. Control cultures showed very little nuclear translocation of NF- $\kappa$ B p65, and treatment with gp120<sub>ADA</sub> was unable to upregulate transcription factor activation, with values similar to control (B). The positive control, Tat, did produce elevations in p65 nuclear translocation, as almost 50% of astroglial cells had distinct nuclear labeling of p65 (\*p < 0.05 vs. control, one-way ANOVA, Tukey's post-hoc); data is based on the mean  $\pm$  SEM of n = 3 experiments.

Figure 2.7



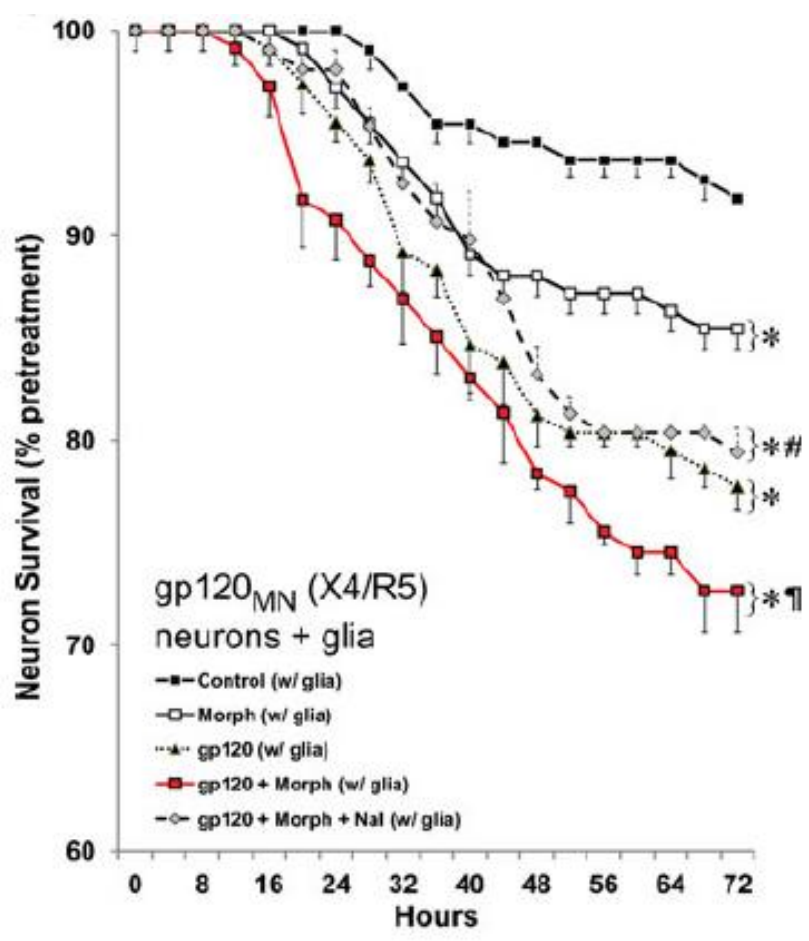


**Figure 2.7** Time-dependent effects of morphine and/or HIV-1 gp120<sub>IIIB</sub> on the survival of medium spiny striatal neurons cultured alone (**a**) or with a glial bedlayer (**b**). There were main effects of both time and treatment, as well as a significant interaction effect (time × treatment) (**a**), which were further examined using Duncan's post-hoc analysis.

Exposure to gp120<sub>IIIB</sub> ± morphine was intrinsically neurotoxic, irrespective of whether neurons were cultured ± glia (**a-b**) (\*  $P < 0.05$  vs. control) (**a**). Morphine transiently and significantly accelerated gp120<sub>IIIB</sub> neurotoxicity above control levels, but only when the neurons were co-cultured with glia (\*\*  $P < 0.05$  vs. control, but not morphine or gp120 treatment, at 12 h;  $n = 6$  experiments) (**b**). Concurrent exposure to naloxone (Nal) negated the combined neurotoxic effects of morphine and gp120<sub>IIIB</sub> at 12 h (**b**).

Interestingly, morphine alone displayed modest, but significant neurotoxicity, but again, only in the presence of high-density glia (\*  $P < 0.05$  vs. control) (**b**). Neuron death is reported as the percentage dying relative to pretreatment numbers; data are the mean number of surviving neurons ± SEM from  $n = 4-6$  experiments. Figure taken from (Podhaizer et al., 2012), with the experiments performed by co-first author Shiping Zou.

Figure 2.8



**Figure 2.8** Time-dependent effects of exposure to morphine (Morph), X4/R5-tropic HIV-1 gp120<sub>MN</sub> and/or naloxone (Nal) in neuron and mixed-glial co-cultures for 72 h. There were significant time and treatment effects for dual-tropic gp120<sub>MN</sub>, as well as a significant interaction effect. Post hoc analyses showed that all treatment groups were different from control values starting at 20 h following continuous exposure, except for morphine, whose effects began at 40 h (\* $P < 0.05$  vs. control). Additionally, morphine co-treatment enhanced the effects of gp120<sub>MN</sub> at both early time-points (20-28 h) and at 56 h and thereafter ( $^{\dagger} P \leq 0.05$ ), and the effects of morphine were prevented by naloxone (Nal) ( $^{\#} P \leq 0.05$  vs. morphine + gp120<sub>MN</sub>; \*  $P < 0.05$  vs. control). Unlike gp120<sub>ADA</sub>, co-administering morphine exacerbated the neurotoxicity of gp120<sub>MN</sub> suggesting that morphine interacts differently with gp120 variants from different HIV-1 strains. Data are the mean number of surviving neurons  $\pm$  SEM from  $n = 4-6$  experiments. Figure taken from (Podhaizer et al., 2012), and experiment performed by co-first author, Shiping Zou.

## **Chapter 3**

# **CCR5 acts as a convergence point for morphine and HIV-1 Tat-mediated neurodegeneration**

### **Introduction**

Despite the advent of combined antiretroviral therapy (cART), which has dramatically increased the life-span of HIV-1 infected individuals and exerted tight control over viral replication (Ray et al., 2010), treatment has not been able to reduce the prevalence of HIV-1 associated neurocognitive disorders (HAND) or the pathological correlates (Sacktor et al., 2002; Cysique et al., 2004; Kramer-Hammerle et al., 2005; Langford et al., 2003; Bell, 2004). This, in an environment where virus is almost absent, begs the exploration into the mechanisms behind the unreconciled degenerative effects, which are hypothesized to result from the persistence of a chronic low level of inflammation (Canestri et al., 2010). In addition to the effects of HIV-1 alone, co-morbid injection drug use worsens the decline in neurocognitive assessments (Carrico et al., 2011). Opioid abuse, in particular, exacerbates CNS inflammatory pathology, known as HIV-1 encephalitis (HIVE), aggravates neurocognitive outcome measures, and increases progression to AIDS (Nath et al., 2002; Kopnisky et al., 2007; Bell et al., 2002; Anthony et al., 2005; Smith et al., 2014). Because viral titer is negligible, it is believed that independent of infection, secreted viral proteins such as Tat, gp120, etc., may be involved in maintaining the inflammatory state of the CNS (Johnson et al., 2013), and it is the downstream effects of these HIV-1 proteins in addition to their potential interactions with opioids that should be targeted.

While interactions of opioids and gp120 are limited and strain-dependent (Podhaizer et al., 2012), previous studies in our laboratory have identified that the HIV-1 Tat protein, in combination with morphine, causes synergistic neurotoxicity in the striatum (Gurwell et al., 2003; Zou et al., 2011). Additionally, this effect only occurs in the presence of glia, and is prevented by use of MOR KO glia (Zou et al., 2011). Identification of points of interaction between HIV-1 Tat and opioid signaling is important to understanding the vulnerabilities for opioid-abusing HIV-1 infected patients. We have chosen to examine the role of the chemokine receptor, CCR5, whose ligand, RANTES, has been shown to be involved in Tat-mediated inflammatory signaling (El-Hage et al., 2008a; El-Hage et al., 2008b), and whose interactions with the opioid receptor, either through formation of heteromers or overlapping signaling mechanisms can significantly modify the response (Chen et al., 2004; Chen et al., 2007; Szabo et al., 2003; Szabo et al., 2002). Additionally, CCR5 is a current target of the adjunct cART therapeutic, maraviroc, which is marketed as an entry inhibitor (Pfizer Inc., 2007; Latinovic et al., 2009). Using both pharmacological inhibition of CCR5 as well as isolation of neural cells from CCR5 WT and KO mice, we aimed to determine 1) if maraviroc could have utility in alleviating bystander effects of HIV-1 infection, 2) whether blockade/deletion of CCR5 could modulate neuronal survival and changes to architecture in Tat and morphine-treated cells, and 3) some of the underlying mechanisms involved to determine if maraviroc or another target of CCR5 may be able to alleviate not only viral entry/infection, but neurocognitive impairments by reducing inflammation and neuronal damage/death. We determined that, in fact, CCR5 is a convergence point of Tat and morphine-mediated neurodegeneration. CCR5 inhibition affects the glial inflammatory

response by reducing NF- $\kappa$ B activation and chemokine release. These data suggest that CCR5 could be a target for the opioid-abusing HIV-1 infected population to prevent neurocognitive decline.

## **Materials and Methods**

Maraviroc and CTAP were purchased from Sigma-Aldrich (St. Louis, MO). The  $\beta$ -chemokines, MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES, were purchased from R&D Systems (Minneapolis, MN). Mice used in this study included ICR, obtained from Charles River Laboratories (Wilmington, MA), and C57BL/6J or CCR5 knockout mice on the C57 background, both of which were purchased from The Jackson Laboratory (Cat. # 000664 and 005427 respectively; Bar Harbor, ME).

### **CC chemokine receptor 5 knockout mice**

CC chemokine receptor 5 knockout mice (and corresponding C57BL/6J control mice) were purchased from The Jackson Laboratory (Bar Harbor, ME). The entire coding region, including the single exon for CCR5 was completely removed and replaced by a neomycin resistance cassette (Kuziel et al., 2003). Verification of CCR5 knockdown was performed using polymerase chain reaction (PCR) with primers against both the wild-type gene: 5'-CAGGCAACAGAGACTCTTGG-3' and 5'-TCATGTTCTCCTGTGGATCG-3' and the mutant gene (primers detect neomycin cassette): 5'-CTTGGGTGGAGAGGCTATTTC-3' and 5'-AGGTGAGATGACAGGAGATC-3' obtained from Integrated DNA Technologies (IDT; Coralville, IA). DNA was extracted from tail-clips using the DNeasy Blood and Tissue extraction kit (Qiagen Inc., Valencia,

CA) according to the manufacturer's instructions. PCR reactions were performed according to the supplier's instructions (The Jackson Laboratory) using Jumpstart Taq polymerase and other reagents from Sigma (St. Louis, MO). PCR products were separated on a 1.5% agarose gel and visualized using ethidium bromide on a Kodak Image Station 440CF.

### **Striatal mixed-glia culture**

P0-P1 imprinting control region (ICR; CD1), C57BL/6J (Cat. # 000664), and/or B6.129P2-Ccr5<sup>tm1Kuz</sup>/J (CCR5<sup>-/-</sup>) pups were utilized for this technique, and cultures were performed according to (Zou et al., 2011b; El-Hage et al., 2005). Striata were dissected from the cerebra, minced, and incubated with trypsin (2.5 mg/mL; Sigma) and DNase (0.015 mg/mL; Sigma) in Dulbecco's modified Eagle's medium (DMEM; Gibco/Invitrogen, Carlsbad, CA) with the following supplements (all from Invitrogen): glucose (27 mM), Na<sub>2</sub>HCO<sub>3</sub> (6 mM) and penicillin/streptomycin for 30 min. at 37°C and 5% CO<sub>2</sub> with intermediate mixing. Tissue was centrifuged at 1000 × g for 5-10 min., media was decanted and tissue was resuspended in DMEM supplemented with glucose, Na<sub>2</sub>HCO<sub>3</sub>, penicillin/streptomycin (100 U/mL and 100 µg/mL, respectively), and fetal bovine serum (10% v/v, Hyclone, Logan, UT). Tissue was triturated through decreasing bore pipettes, filtered sequentially through 100 and 40 µm nylon mesh pores, and plated on poly-L-lysine coated tissue culture plates alone or with coverslips at 150 × 10<sup>5</sup> cells/cm<sup>2</sup>. Cells were grown in 10% FBS-containing DMEM for 10-14 days with media exchanged on day 1 and then every 3 days subsequently until confluence was reached.

### **Striatal neuron culture**

E15-16 mouse embryos were removed from the dam, striata dissected, and tissue incubated in trypsin (2.5 mg/mL) and DNase (0.015 mg/mL) diluted in neurobasal medium (NBM) containing B27 (1x; Invitrogen), glutamate (0.025 mM; Sigma-Aldrich Co.), L-glutamine (0.5 mM; Invitrogen), and penicillin/streptomycin (100 U/mL and 100 µg/mL, respectively) for 30 min. at 37°C and 5% CO<sub>2</sub>. Tissue was centrifuged at 1000 × g for 5-10 min., resuspended in fresh media, triturated and filtered through 70 µm nylon mesh pores. Cells were plated either on poly-L-lysine coated cell culture plates or on top of a confluent layer of mixed-glia. Neurons and co-cultures containing neurons and mixed-glia were maintained in the above NBM concoction, and fed on day 1 and every two days thereafter for 7-10 days.

### **Cell treatments**

ICR cells were treated with morphine sulfate (500 nM; Sigma-Aldrich Co.), HIV-1 Tat<sub>1-86</sub> (Immunodiagnosics, Inc.; Woburn, MA), or morphine and Tat combined alone or pretreated with MVC (50 nM; Y. Sigma-Aldrich Co.) and/or CTAP (500 nM; Sigma). Additional studies involved cells being treated with CCR5 ligands, RANTES, MIP-1α, or MIP-1β at concentrations ranging from 0.1 nM to 1.0 µM. C57 and CCR5<sup>-/-</sup> cells were treated with morphine, Tat<sub>1-86</sub>, or the combination, and occasionally CTAP was used to ensure that opioid function was occurring through MOR. Mixed-glia were treated in supplemented DMEM lacking FBS, while neurons and neuron mixed-glia co-cultures were treated in glutamate-free supplemented NBM.



### **Repeated measures time-lapse microscopy**

Computer-aided video recordings of individual neurons were made using time-lapse microscopy on a Zeiss Axio Observer Z1 inverted microscope (Carl Zeiss Inc.). Eight fields of neurons were sampled every 30 min. for a period of 72 h using an automated, computer-controlled stage encoder and Axiovision 4.6 software (Carl Zeiss Inc.), with cells maintained at 37°C in 5% CO<sub>2</sub> using an XL S1 environmental incubator (PeCon). Preselected neurons were examined for viability over the time-course of the experiment and neuron survival as a percent of pretreatment was binned at 4 h intervals and analyzed by two-way repeated measures ANOVA with Duncan's post hoc analysis (Statistica, StatSoft). Rigorous morphological criteria were used to determine neuron viability including fragmentation and/or involution of the cell body, which has been confirmed previously by staining/fluorescent labeling previously (Buch et al., 2007). Data represent mean  $\pm$  SEM of n = 4 independent experiments.

### **Immunocytochemistry**

As an overview, cells were fixed in 4% paraformaldehyde and permeabilized in 1X PBS with 0.1% Triton-X-100, both for 15 min. at rm. temp. Cells were then blocked in 1X PBS with 0.1% BSA and 2% goat serum, 1 h at room temperature, and incubated in primary antibodies overnight at 4 °C. Cells were washed in three exchanges of fresh PBS at 15 min. intervals and incubated in secondary antibody for 1 h at room temperature. When multiple primary and secondary antibodies were used, addition of the primary and therefore secondary antibodies as well was done sequentially to

prevent cross-reactivity. Cells were counter-stained with 0.5 µg/mL Hoechst 33342 (Invitrogen) for 15 min., and rinsed 3 × 15 min. and mounted on coverslips using Prolong Gold Antifade Reagent (Invitrogen) and dried overnight. Cells were imaged as indicated in the specific experimental methods sections.

### **Neurite measurement**

Following 72 h treatment, neuron mixed glial co-cultures were prepared for immunocytochemical analysis by labeling with mouse anti-microtubule associated protein 2 (MAP2; Chemicon, MAB378; 1:1000) followed by goat-anti mouse 594 (Invitrogen). Ten images of each treatment were taken using a 20 x objective, and total neurite length was assessed using a modified Sholl analysis (Figure 3.1) (SHOLL, 1953; Hauser et al., 1989; Singh et al., 2005) with at least 50 neurons per treatment being measured. Sholl analysis measures were converted to µm for statistical analysis. Results are the mean ± SEM of n = 3 - 4 independent experiments.

### **Glial distribution**

Following 72 h treatment, mixed-glia cultures were labeled with rabbit anti-Iba-1 (1:200; Wako, Osaka, Japan) and mouse anti-GFAP (1:750; Millipore, AB5804) primary antibodies followed by goat anti-rabbit and goat anti-mouse secondary antibodies, respectively. Five images of each coverslip were taken at 40 x. The numbers of GFAP-immunopositive cells and Iba-1 immunoreactive cells were quantified in n = 3 independent experiments.

## **ELISA assessment**

ICR, C57BL/6J, and CCR5<sup>-/-</sup> cells were treated for 12 h, after which, conditioned media was removed and stored at -80°C until further use. Levels of cytokines and chemokines were assessed using kits for TNF- $\alpha$  and RANTES (R&D Systems); and MCP-1 (BP Opteia) and results were based on a standard curve.

## **Statistics**

All data was presented as mean  $\pm$  SEM unless otherwise noted. For time-lapse studies, a repeated measures analysis of variance (ANOVA) was performed followed by Duncan's post hoc analysis if applicable. For all other studies, two-way ANOVAs were used with Duncan's post hoc analysis where appropriate.  $p < 0.05$  was considered significant.

## **Results**

### **Maraviroc prevents interactive neurotoxicity of morphine and Tat**

Repeated measures analysis of neuronal survival over 72 h greatly increases the sensitivity of the technique (Figure 3.3). In ICR cultures, both morphine and Tat exhibited significant toxicity (\* $p < 0.05$  vs. control), while an enhancement in the toxic effects was noted when morphine and Tat were added in combination (\*\* $p < 0.05$  vs. control; \$ $p < 0.05$  vs. morphine or Tat). Pretreatment with 50 nM maraviroc had no significant effect on basal neuron viability, nor did it alter the responses of neurons to morphine or Tat individually. However, this concentration of maraviroc prevented the interactive effects of morphine and Tat, suggesting that CCR5 indeed is involved in the

striatal response to morphine and Tat (\*p < 0.05 vs. control; #p < 0.05 vs. morphine + Tat). In other studies (not shown), maraviroc's effects were not concentration-dependent in the nanomolar range and literature from Pfizer suggests cross-reactivity with MOR occurs around 100 nM, so concentrations 100 nM and above were not used (Pfizer Inc., 2007).

### **Genotypic changes in combination with drug treatment alter the distribution of glia**

Figure 3.4 shows cell counts after 72 h for cells treated with morphine, Tat, CTAP, or a combination thereof. Under control conditions, there was no difference in immunopositivity of GFAP and Iba-1 labeling. However, when morphine and Tat were treated alone, or when not combined but in the presence of CTAP, CCR5<sup>-/-</sup> cultures had significantly more Iba-1 immunoreactive cells compared to C57BL/6J. However in the presence of morphine and Tat, or morphine and Tat and CTAP, there was no difference in the glial cell distribution between genotypes. In C57BL/6J mice, morphine + Tat treatment caused an increase in Iba-1 immunolabeling.

### **Combinations of C57BL/6J and CCR5<sup>-/-</sup> modify the response to Tat and morphine**

(A) C57BL/6J glial / C57BL/6J neurons. Neurons and glia cultured from a C57 background (Figure 3.5 A,B) showed similar responses to their ICR counterparts (Figure 3.3). Morphine and Tat alone caused neurotoxicity after 72 h (\*p < 0.05 vs. control), and the effect of the combination treatments was enhanced compared to morphine or Tat alone (\*\*p < 0.05 vs. control; \$p < 0.05 vs. morphine or Tat). Additionally, CTAP

pretreatment was employed to show that the augmentation of Tat-mediated toxicity by morphine was through MOR, which it appears to be, as it is able to return neuron survival to levels of Tat alone ( $\#p < 0.05$  vs. morphine + Tat). (B) CCR5<sup>-/-</sup> glia / C57BL/6J neurons (Figure 3.5 C,D). The absence of CCR5 in glia prevented toxic responses to both morphine and Tat alone, and there was also no effect of the combination treatment. (C) C57BL/6J glia / CCR5<sup>-/-</sup> neurons. Interestingly, in cultures containing neurons lacking CCR5, morphine and Tat toxicities alone were preserved ( $*p < 0.05$  vs. control), but in this case, the morphine and Tat combination treatment failed to produce an effect. (D) CCR5<sup>-/-</sup> glia / CCR5<sup>-/-</sup>. Basal conditions with this combination of cells produced significant toxicity compared to the C57BL/J glia/ CCR5<sup>-/-</sup> control group which was run at the same time ( $\$p < 0.05$  vs. matched control). However, under these conditions, treatment with neither morphine nor Tat was able to alter the level of toxicity.

### **Decline in neurite length is alleviated by maraviroc pretreatment**

Studies were conducted at 72 h to assess changes in neurite length using Sholl analysis to determine overall neurite length by the number of interactions a particular neuron makes with a circular grip. Morphine did not have a significant effect on neurite length at this time point. However, morphine + Tat did caused a slight decrease in overall neurite length ( $*p < 0.05$  vs. control; Figure 3.6). Pretreatment with 50 nM maraviroc prevented the change in these two treatment groups, while maraviroc had no effect on its own.

### **Glial and neuronal CCR5 knockout cell combinations show differential effects in response to Tat or morphine + Tat**

In C57BL/6J glia and neurons, application of morphine + Tat produced a significant decrease in neurite length ( $\mu\text{m}$ ) (\* $p < 0.05$  vs. control; Figure 3.7). This decline in neurite length was not noted with CCR5<sup>-/-</sup> cells were cultured with C57BL/6J neurons. In cultures composed of C57BL/6J glia and CCR5<sup>-/-</sup> neurons, there was no effect of treatment on neurite length, however when CCR5 was absent in both neurons and glia, the basal neurite length was shorter than if CCR5 was present on either neurons or glia (\* $p < 0.05$  vs. control).

### **NF- $\kappa$ B p65 nuclear translocation is attenuated by maraviroc pretreatment**

P65 nuclear translocation, as assessed by localization of the p65 antibody to the nuclear compartment was assessed in 10 fields of astrocytes at 63x magnification (Figure 3.8 A). Treatment with Tat or morphine and Tat significantly induced p65 nuclear translocation, which was dampened by pretreatment with maraviroc. However, numbers of cells immunopositive for nuclear p65 following maraviroc treatment was still significant from control, suggesting that maraviroc is unable to block the process entirely, but may be able to inhibit a point in the downstream pathway.

### **Chemokine secretion is reduced by maraviroc**

Conditioned medium was removed from treated cells 12 h following treatment and frozen at  $-80^{\circ}\text{C}$  until use. ELISAs for TNF- $\alpha$ , RANTES, and MCP-1 were performed according to the manufacturer's protocol (Figure 3.8 B). Treatment with Tat or Tat +

morphine produced significant increases in cytokine and chemokine secretion, which has been established previously. Pretreatment with maraviroc had no effect on TNF- $\alpha$  release, but did suppress both RANTES and MCP-1 secretion.

### **CCR5 knockout glia have attenuated secretion of chemokines**

C57BL/6J glia were able to secrete significant levels of the cytokine TNF- $\alpha$ , RANTES, and MCP-1 in response to Tat or morphine + Tat, with no difference noted between the treatments (\*\* $p < 0.05$  vs. control; Figure 3.9). CCR5<sup>-/-</sup> glia treated with Tat or morphine + Tat secreted equivalent TNF- $\alpha$  to wild-type cells, but the release of the chemokines RANTES and MCP-1 was significantly dampened compared to Tat or morphine + Tat (<sup>#</sup> $p < 0.05$  vs. control). Morphine showed very little effect in this assay.

### **Time-lapse studies on the CCR5 ligands, MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES produce modest toxicity**

Concentration-response studies were undertaken to determine the extent to which  $\beta$ -chemokines could activate neurotoxic signaling pathways in neuronal mixed-glial co-cultures. Cells assayed for 48 h showed modest toxicity in response to  $\beta$ -chemokine ligands (Figure 3.10), and neurotoxic responses were not concentration-dependent. Concentrations of 100 pg/mL and 100 ng/mL of MIP-1 $\alpha$  produced significant neurotoxicity, but those concentrations in between were not significantly different (\* $p < 0.05$  vs. control). MIP-1 $\beta$  produced slightly less neuron cell death over the 48 h, which was not significantly different from control conditions, and RANTES produced significant toxicity at 10 ng/mL and 1.0  $\mu$ g/mL.

## **Discussion**

Because of the ineffectiveness of current cART regimens on the development of HAND (Sacktor et al., 2002; Cysique et al., 2004; Kramer-Hammerle et al., 2005) and the exacerbation of neurocognitive symptoms in opioid-abusing populations of HIV-1 infected persons (Carrico et al., 2011), it is critical to explore mechanisms to interfere with these pathways both alone and in combination. Herein we report that maraviroc is able to prevent co-morbid effects of HIV-1 Tat and opioids on striatal neuron survival and on sublethal changes to neurite length. We further show that there are both neuronal and glial components to these effects through selective use of CCR5 knockout cells. It appears that glial inflammatory signaling can explain a part of the pathway involved in interactive neurodegeneration, but the underlying mechanisms of CCR5's actions still need to be explored.

While CCR5 has been implicated in HIV-1 pathogenesis due to its interactions with gp120 molecules, affecting viral entry and infection while mediating aberrant GPCR signaling (Khan et al., 2004; Freedman et al., 2003; Wu and Yoder, 2009), CCR5 has not, until now, been shown to also modulate bystander neurotoxic effects of viral proteins through its indirect actions downstream of HIV-1 Tat. In addition, CCR5 also appears to be a convergence point for opioid-HIV-1 interactions, but only in the case of HIV-1 Tat; R5-tropic gp120<sub>ADA</sub> does not produce interactive neurotoxicity with morphine (Podhaizer et al., 2012).

Both CCR5 antagonism with maraviroc and use of CCR5 knockout cells were able to modify the neurotoxic effects of Tat and morphine, but to different extents. Maraviroc was able to selectively alleviate interactive toxicity, while absence of glial



CCR5 alleviated morphine, Tat, and morphine + Tat toxicity completely. Studies using CCR5 knockout neurons attained similar results to the maraviroc studies. It is known that maraviroc is a poor antagonist in rodents, and that the lack of effect can be, at least in part, attributed to the shorter half-life (Pfizer Inc., 2007). However, in the absence of metabolic function, as occurs in our cell culture system, the potency and efficacy of the drug compared to human cells is not known and it is possible that maraviroc is not acting to its therapeutic potential in our model. Because maraviroc and culture of CCR5 knockout neurons produced similar effects, it is possible that maraviroc has differential affinity for neurons and glia, and that at lower concentrations, maraviroc is binding predominately to neuronal CCR5 to attenuate the interactive neurotoxic response. Use of higher concentrations of maraviroc is not possible due to its inherent toxic effects, so in this way we are not able to extend the studies.

Additionally, that neuronal and glial CCR5 play different roles in morphine and Tat interactions is quite interesting, and may shed some light on how the cells are able to cross-talk in our model. When glia lacking CCR5 are cultured with wild-type neurons, there is no significant toxicity from any treatment, which could imply that glial CCR5 is responsible for the effects of Tat and morphine individually and has no effect on the interaction, or that it is also partially responsible for the integrated signal. Under the opposite conditions, when CCR5 is absent only from neurons, a very different response is seen. It appears that neuronal CCR5 is not involved in the individual actions of morphine or Tat, but that it plays a role in signal integration. That morphine's actions require glia (Zou et al., 2011b; Gurwell et al., 2001) was confirmed by these studies and suggest further that glial CCR5 is sufficient to facilitate this effect. Additionally that

morphine and Tat interactions were blocked by CTAP, suggests that these effects are MOR-mediated. Whether morphine and Tat combine in glia to create a unique signal to which neurons are sensitive or whether the signal integration occurs in the neurons remains to be examined. Additionally, it was interesting to note that CCR5 appears to be required under physiological conditions for neuron survival. And it is surprising that maintenance of this chemokine tone is so important, but doesn't appear to depend on which cell has the receptor, only that it is present. Researchers are now, with more frequency, examining physiological roles for chemokines, though the majority of the research thus far has been on CXCR4/SDF-1 signaling, which is critical to neuronal development and positioning within the CNS (Bagri et al., 2002) and lack of either ligand or receptor proves lethal in neonates (Ma et al., 1998; Zou et al., 1998). It has been suggested that CCR5 may too have neurotrophic effects, aiding DRG cell migration (Bolin et al., 1998) and neuronal connection development (Westmoreland et al., 2002; Geppert, 2003; Rezaie et al., 2002), though there is currently little evidence to support this claim.

Neurotoxic effects of HIV-1 proteins and opioids can be assessed under very sensitive conditions using stringent criteria, however, in vivo, HIV-1 proteins and opiates cause neuronal damage, but rarely produce neurotoxicity (Bellizzi et al., 2006; Kim et al., 2003; Sa et al., 2004), even in those with moderate to severe forms of HAND. The neurotoxicity studies generally correlate with other forms of examination, but we extended our studies to address sublethal changes to neurite length and performed studies to both examine maraviroc's ability to modify the response of morphine and Tat, alone and in combination. In this measure, results mimicked those of the toxicity studies

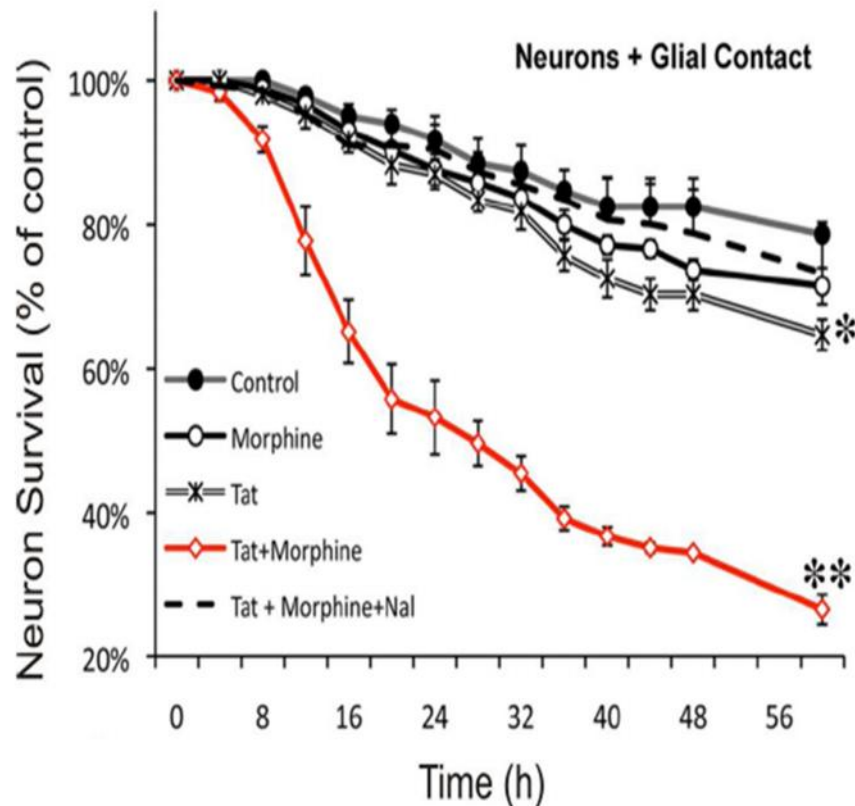
but for a few differences: under these conditions, in the wild-type neuron and glial cultures, only Tat + morphine were able to cause decreases in neurite length, and no treatment effect was noted in cultures composed of wild-type glia and CCR5 knockout neurons.

Maraviroc inhibition of CCR5 produced similar effects to cultures of CCR5-deficient glia, with the ability to modify chemokine secretion but lacking the strength to alleviate the increase in chemokines to levels of control or to alter the release of early-response cytokines, suggesting that this pathway may influence glial inflammatory cascades leading to neurodegeneration and death, but that this is not the only mechanism. That the  $\beta$ -chemokine ligands themselves produced little toxicity to co-cultures suggests that the pathway(s) stimulated by these proteins alone may be different than those when Tat has primed the system and that only together with other signals do they become neurotoxic.

It is unclear how to separate CCR5 and its ligands' paradoxical roles in HIV-1 infection and neuroinflammation in general. In diseases such as HIV-1 and viral plagues, blockade of CCR5 or its absence due to the  $\Delta 32$ -CCR5 genetic mutation are protective (Kantarci et al., 2005; Duncan et al., 2005). However, CCR5 is critical in one's ability to combat West Nile Virus and Japanese Encephalitis (Larena et al., 2012). Additionally, while CCL5/RANTES can be involved in mounting an immune response (El-Hage et al., 2008a), its extracellular presence competitively inhibits viral entry and aberrant signaling by co-receptors (Olson et al., 1999; Madani et al., 1998; Kaul et al., 2007; Avdoshina et al., 2010).

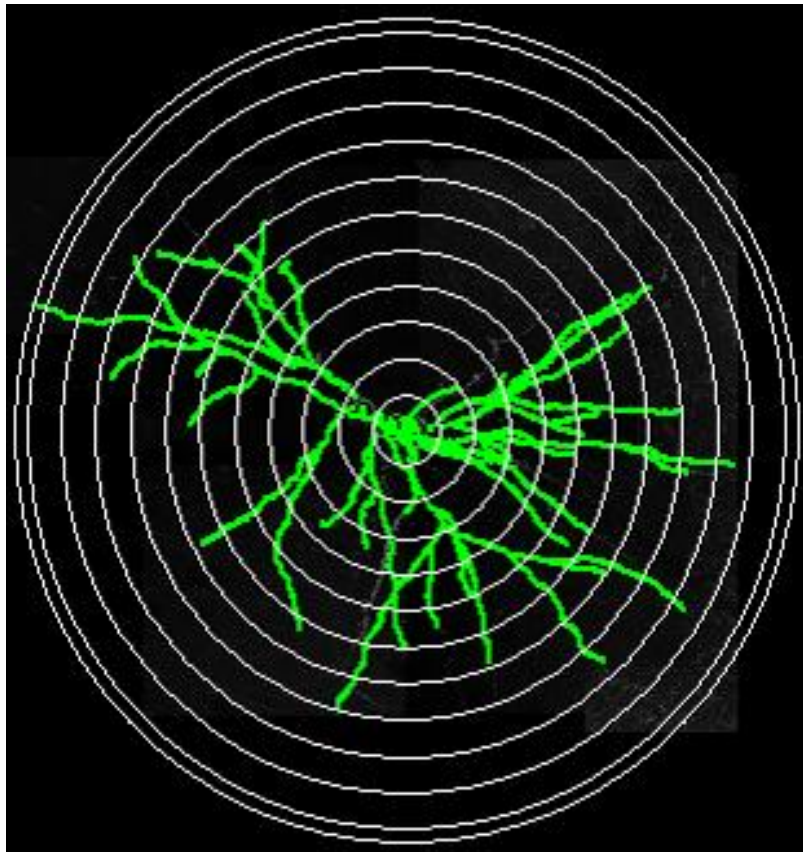
In our studies, the rescue of neurotoxicity despite high levels of CCR5 ligand could have positive implications for neurocognitive outcomes, as higher CCL5 levels in CSF correlated with neuropsychological performance (Letendre et al., 1999), and suggests that inhibition of CCR5 with maraviroc may be modulating the negative consequences of CCR5-chemokine interactions while preserving the pro-survival aspects of treatment.

**Figure 3.1**



**Figure 3.1** Neurotoxic interactions between Tat and morphine are mediated by glia. Striatal ICR neurons were co-cultured with mixed glia of the same region and the different cell types were allowed in physical contact with one another. Tat by itself caused significant toxicity compared with untreated controls at 40-60 h (\*  $P < 0.05$  double lines). When Tat and morphine exposure were combined, the reduction in neuron survival was dramatic. The combination of Tat + morphine was toxic at 8 h and at all time points thereafter versus all other groups (\*\*  $P < 0.05$ , red line). Naloxone blocked the synergistic effects of morphine and brought survival to control levels.

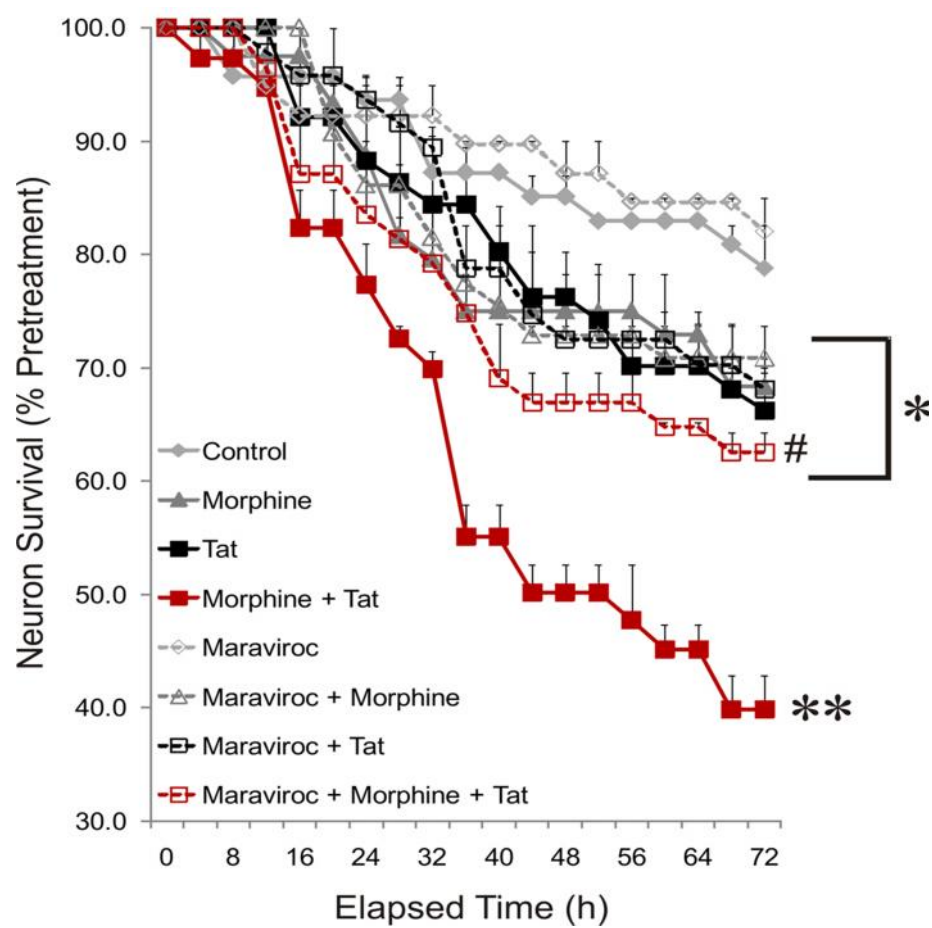
**Figure 3.2**



<http://research.mssm.edu/cnic/help/ns/analysis.html>

**Figure 3.2** A modified Sholl analysis was performed by first placing the center of the grid of concentric circles over the neuron cell body. Total neurite length of a neuron was quantified based on the sum of the number of intersections with the circles of the grid of all the neurites. In order for an intersection to be counted, a neurite had to have grown past the concentric circle. After the number of intersections had been documented, this score was converted to microns based on a multiplication factor obtained based on the distance between the concentric circles on the grid and the distance between lines on a hemocytometer under the same magnification to achieve an interpretable value.

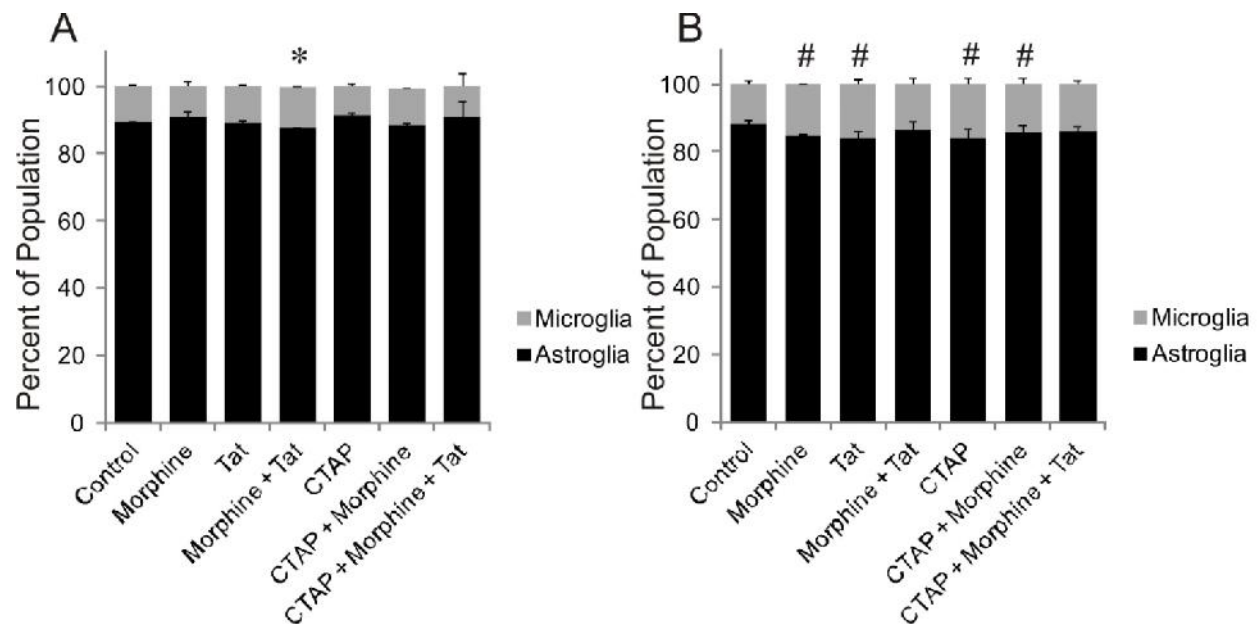
Figure 3.3



**Figure 3.3** Time-dependent effects of morphine and/or Tat on neurotoxicity in the presence or absence of the CCR5 antagonist, maraviroc. Neuron mixed-glial co-cultures from ICR mice were treated with morphine (500 nM), Tat (100 nM), and maraviroc (50 nM) alone or in combination and assessed by repeated measures time-lapse microscopy for neuron survival as a percent of pretreatment over 72 h in 4 h time bins. Morphine and Tat caused significant neurotoxicity on their own (\* $p < 0.05$  vs. control) with a significant increase in toxicity with morphine and Tat combined (\*\* $p < 0.005$  vs. control; \$ $p < 0.05$  vs. morphine or Tat). Maraviroc pretreatment did not significantly differ from control, nor did it prevent morphine or Tat toxicity, but it did interfere with the interactive effect of morphine and Tat (# $p < 0.05$  vs. morphine + Tat). Values are the mean  $\pm$  SEM of  $n = 4$  experiments; two-way ANOVA with Duncan's post-hoc analysis.

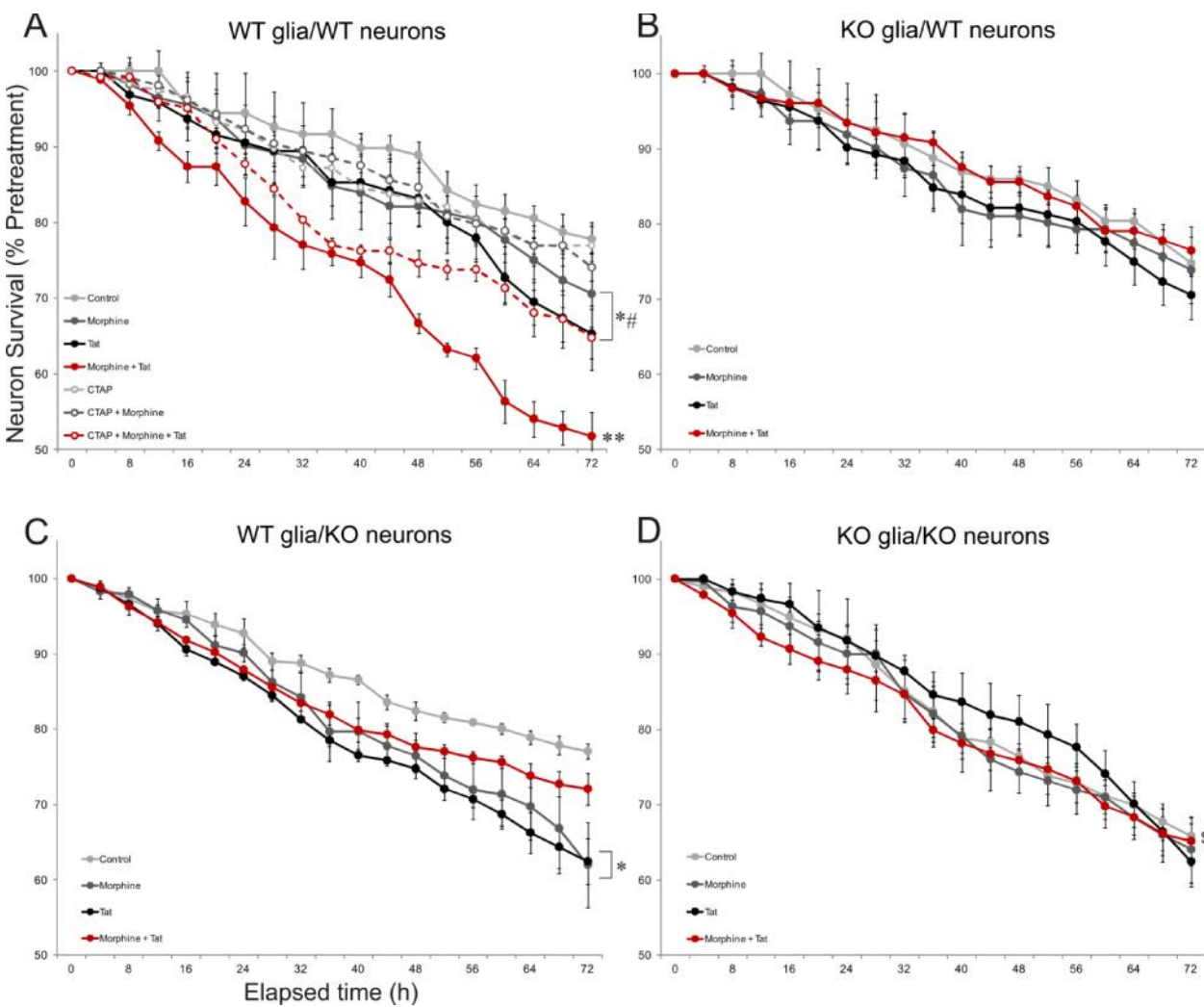


**Figure 3.4**



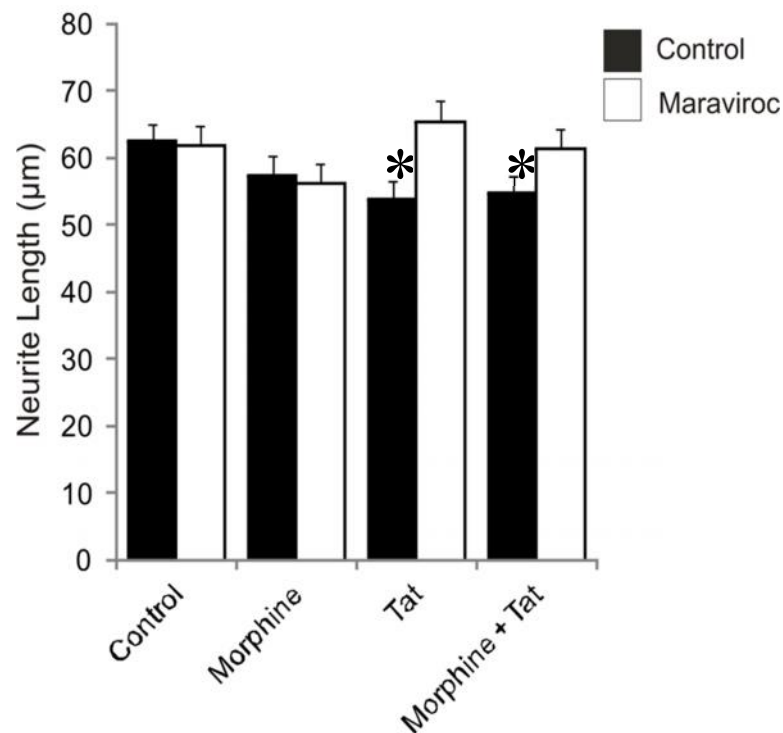
**Figure 3.4** Percentage of cells in mixed-glial cultures of both C57BL/6J and CCR5<sup>-/-</sup> genotypes expressing cell-specific markers after 72 h treatment. Under wild-type conditions (**A**), only the combination of morphine and Tat was able to elevate the number of Iba-1<sup>+</sup> microglial cells from control (\*p < 0.05 vs. control). The distribution of glial cells was not different between genotypes under control conditions, however treatment with either morphine or Tat alone caused a significant increase in Iba-1<sup>+</sup> immunoreactivity in CCR5<sup>-/-</sup> cells (#p < 0.05 vs. respective treatment across genotype; **B**). Morphine and Tat treatments in knockout cells failed to elevate Iba-1 immunopositivity above the wild-type cells, neither did the combination in the presence of CTAP, though CTAP alone and CTAP + morphine did, in fact, show elevated Iba-1<sup>+</sup> cells in CCR5<sup>-/-</sup> mixed-glial cultures. Two-way ANOVA with Duncan's post-hoc test; data are the mean ± SEM of n = 3 experiments.

Figure 3.5



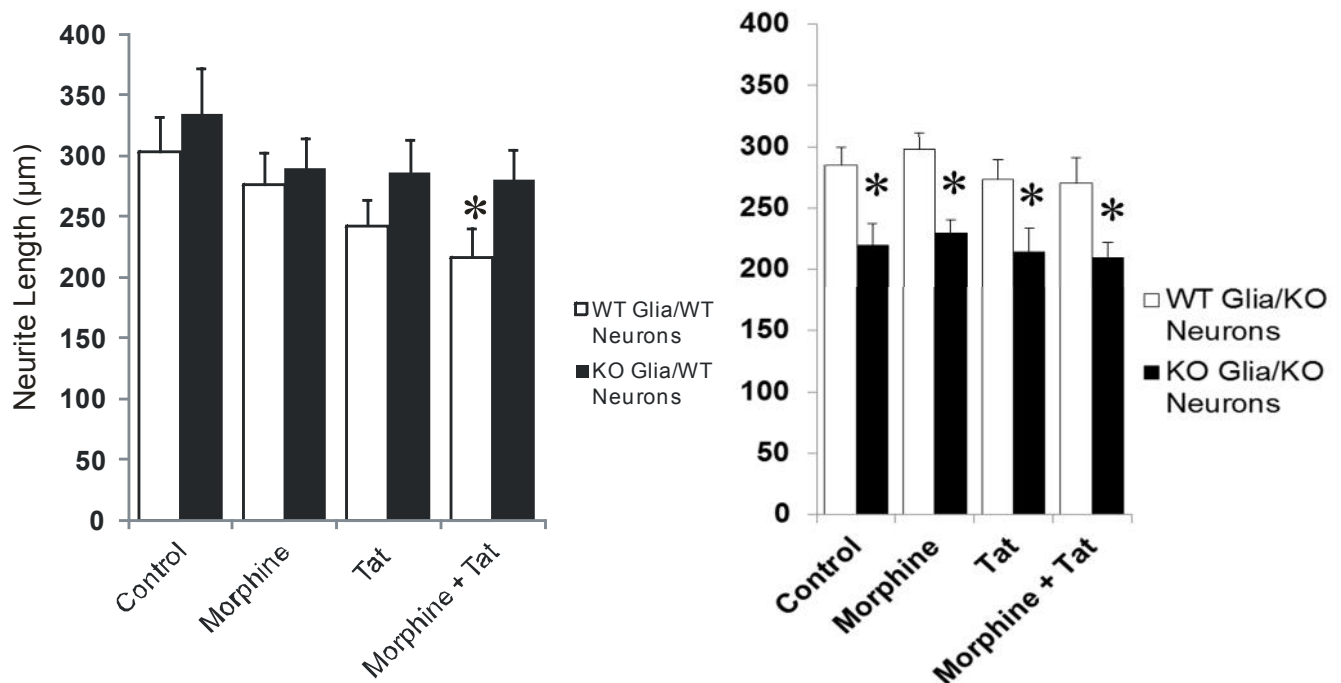
**Figure 3.5** Time-lapse of neurotoxic effects of morphine and Tat alone or treated together in combinations of wild-type and CCR5<sup>-/-</sup> neurons and mixed-glia. A) wild-type glia and neurons showed similar results to their ICR counterparts. Morphine and Tat alone produced a significant increase in neurotoxicity (\*p < 0.05 vs. control), while treatment in combination produced a significant increase in toxicity (\*\*p < 0.005 vs. control), and was more neurotoxic than either morphine or Tat treatment alone (# p < 0.05 vs. morphine + Tat) after 72 h. Pretreatment with CTAP prevented the augmentation of Tat's neurotoxic effects by morphine (#p < 0.05 vs. morphine + Tat), while CTAP had no effect on its own. B) CCR5<sup>-/-</sup> glia cultured with wild-type neurons showed no neurotoxic response to morphine, Tat, or the co-applied treatments after 72 h of treatment. C) In wild-type glia cultured with CCR5<sup>-/-</sup> neurons, treatment with morphine or Tat produced a significant neurotoxic response (\*p < 0.05 vs. control), but when combined, morphine and Tat failed to induce toxicity. D) CCR5<sup>-/-</sup> glial cultured with CCR5<sup>-/-</sup> neurons showed significant neurotoxicity under control conditions (\$p < 0.05 vs. wild-type glia/CCR5<sup>-/-</sup> neuron control), however treatments or morphine, Tat, or dual-application produced no further change to neuron survival. Two-way repeated measures ANOVA followed by Duncan's post hoc analysis; data are the mean ± SEM of n = 4 experiments.

**Figure 3.6**



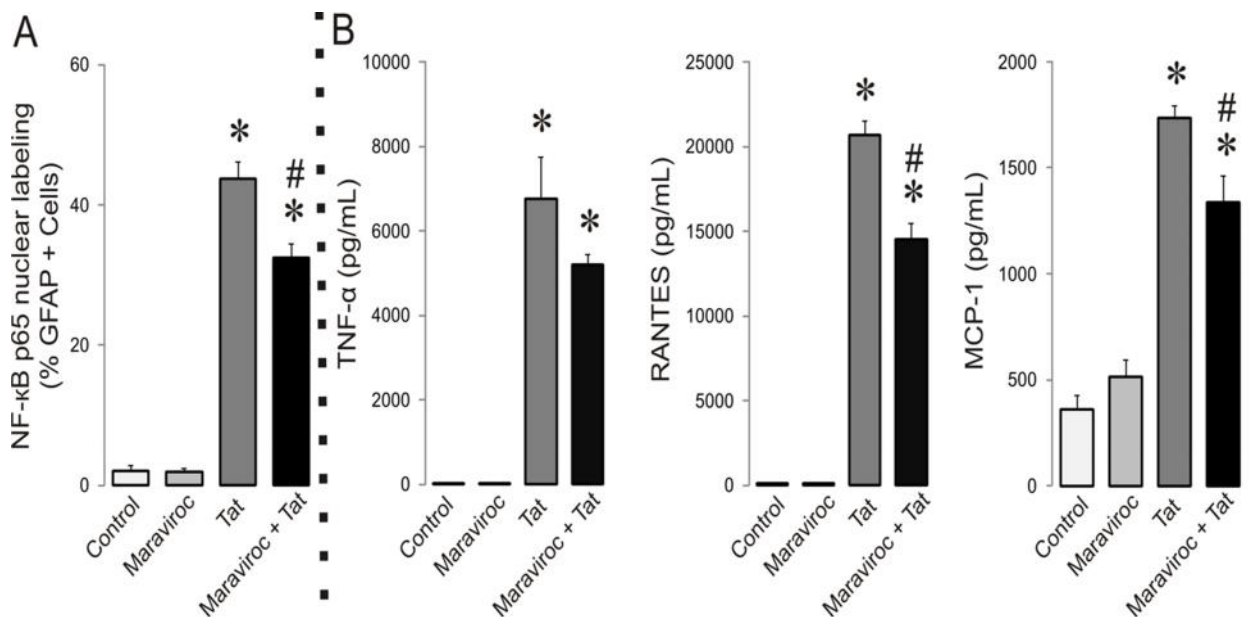
**Figure 3.6** Results of Sholl analysis for neurite length in ICR mice treated with morphine of HIV-1 Tat in the presence of absence of maraviroc. Neuron mixed-glia co-cultures were treated with morphine, Tat, or morphine and Tat for 72 h and neurons were labeled with MAP2. Sholl analysis was performed on 20 images taken at intervals across the coverslip and total neurite length was assessed. Tat and morphine + Tat treatments produced significant decreases in neurite length after 72 h treatment (\* $p < 0.05$  vs. control), while morphine had no effect alone. Pretreatment with maraviroc had no effect alone and prevented the decrease in neurite length by Tat and morphine + Tat. Two-way ANOVA with Tukey post-hoc test; data are the mean  $\pm$  SEM of  $n = 4$  experiments.

**Figure 3.7**



**Figure 3.7** Effects of combinations of wild-type and CCR5<sup>-/-</sup> cells on neurite length after 72 h treatment. Sholl analysis was performed as described in Figure 3.3. A) In cultures composed of wild-type glia and neurons, only treatment with both morphine and Tat produced a simplification of the neurites (\*p < 0.05 vs. control). This effect was blocked in cultures of CCR5<sup>-/-</sup> glia and wild-type neurons, which showed no change in neurite length by treatment. B) Treatment of wild-type glia and CCR5<sup>-/-</sup> neurons for 72 h caused no change in neurite length with treatments of morphine, Tat, or morphine + Tat. Use of both CCR5<sup>-/-</sup> glia and neurons showed a simplification of the neurites under basal conditions, though no additional treatment effects were seen (\*p < 0.05 vs. control). Two-way ANOVAs with Tukey post-hoc test; data are mean ± SEM of n = 4 experiments.

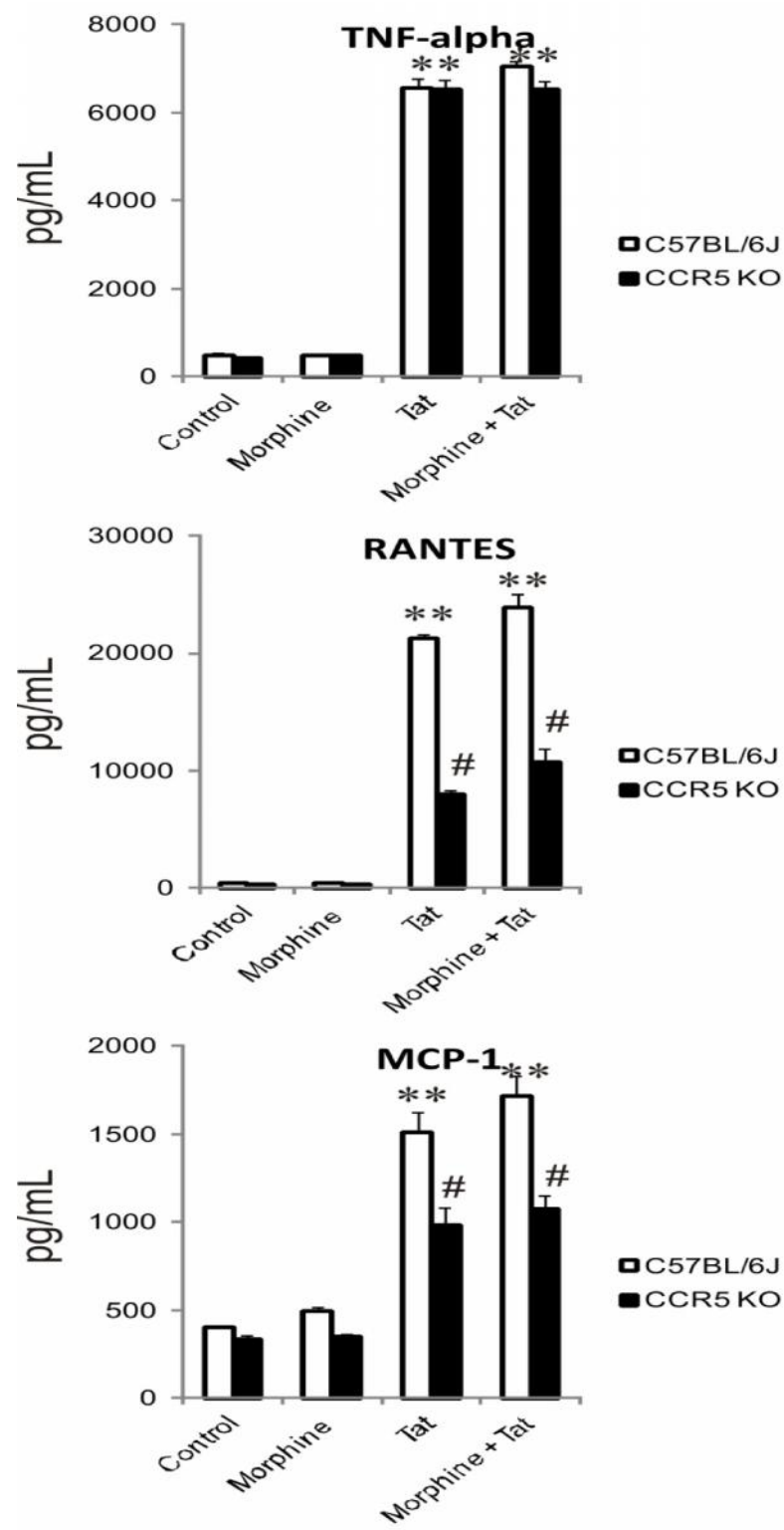
**Figure 3.8**



**Figure 3.8** Effects of NF-κB p65 nuclear translocation and cytokine/chemokine release in glia after 12 h of treatment. A) Following treatments, mixed-glia grown on coverslips were labeled with NF-κB p65 and GFAP antibodies to identify the transcription factor in astroglial cells. Application of Tat or morphine + Tat significantly produced a significant induction of nuclear translocation of p65 as compared to control (\*p < 0.05 vs. control), but were not different from one another. Pretreatment of Tat or morphine + Tat treated cells with maraviroc significantly attenuated p65 induction, but was still elevated above control levels (\*p < 0.05 vs. control; #p < 0.05 vs. Tat or morphine + Tat, respectively).

B).

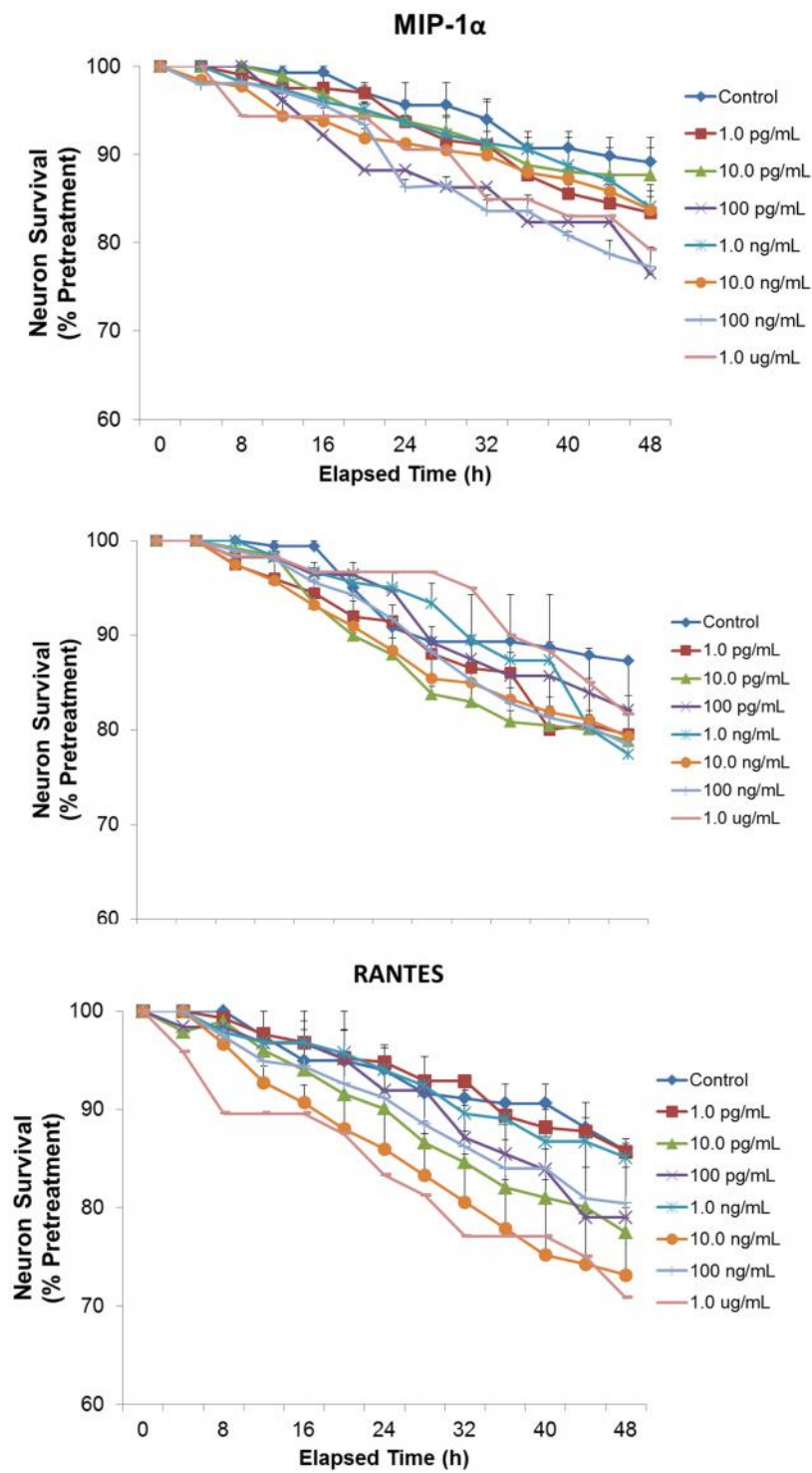
Figure 3.9



**Figure 3.9** Results of application of morphine, Tat, or morphine + Tat onto wild-type mice or CCR5<sup>-/-</sup> mice. Mixed glial cultures of either wild-type or CCR5 knockout origin were treated with opioid and HIV-1 protein for 12 h, and conditioned medium was removed and stored at -80°C until use. ELISA analyses were performed to quantify the amount of TNF- $\alpha$ , RANTES, and MCP-1 secreted into the medium during the treatment period. In wild-type cultures, Tat or Tat + morphine treatment were able to upregulate secretion of the cytokine TNF- $\alpha$  and the chemokines RANTES and MCP-1, but Tat and Tat + morphine were not found to be different from one another (\*\*p < 0.005 vs. control). In CCR5<sup>-/-</sup> mixed glial cells, TNF- $\alpha$  production was no different from the level of its genetic control, though both levels of RANTES and MCP-1 were significantly attenuated (<sup>#</sup>p < 0.05 vs. corresponding wild-type treatment). Two-way ANOVA with Tukey post hoc analysis; data shown are the mean  $\pm$  SEM of n = 3 experiments.



Figure 3.10



**Figure 3.10** Assessment of the neurotoxic response of CCR5's  $\beta$ -chemokine ligands in neuron mixed-glial co-cultures. Concentration-dependent assessments of chemokine ligands between 1.0 pg/mL and 1.0  $\mu$ g/mL were performed over a 48 h treatment period and assessed for neurotoxicity in our mixed culture paradigm. Toxicity levels were low across all treatments, and the ability of a chemokine to cause cell death did not appear to be concentration-dependent. MIP-1 $\alpha$  produced significant neurotoxicity after 48 h at 100 pg/mL and 100  $\mu$ g/mL, but interestingly not at the concentrations in between (\*  $p < 0.05$  vs. control). MIP-1 $\beta$  treatment did not induce significant neurotoxicity, while RANTES produced a significantly toxic response at 10 ng/mL and 1.0  $\mu$ g/mL of ligand (\*  $p < 0.05$  vs. control). Two-way repeated measures ANOVA followed by Duncan's post hoc test; data are the mean  $\pm$  SEM of  $n = 3$  experiments.

## **Chapter 4**

### **Behavioral effects of Tat induction and morphine administration in a Tat-transgenic mouse model**

#### **Introduction**

As the life-span of HIV-1 infected individuals increases due to the use of cART therapy, the incidence of HIV-1 associated neurocognitive disorders is also on the rise (McArthur et al., 2010). These changes have occurred in the absence of a detectable viral load in the CNS, suggesting that viral components/proteins, but not the virus itself is likely responsible for changes in neurocognition (Johnson et al., 2013). Secreted virotoxins, such as HIV-1 Tat, are able to cause pathological changes to the CNS through direct and indirect injury to neurons (Jin et al., 2012;Fitting et al., 2010a;Zou et al., 2011b) and the activation of glia (Bokhari et al., 2009;El-Hage et al., 2008b;El-Hage et al., 2006). Opioid drugs can exacerbate the effects of HIV-1 through interactions specifically at the level of the glia (Zou et al., 2011b) to drive inflammatory signaling cascades and neuron cell death (Zou et al., 2011b;El-Hage et al., 2005). While reports of pathological changes in the opioid abusing HIV-1 infected population are numerous (Bell et al., 1998;Bell et al., 2006;Anthony et al., 2005) examining these interactions clinically is very difficult as most who are abusing substances are poly-drug users, making it difficult to determine the underlying mechanisms of neurocognitive changes. Our Tat-transgenic mouse model is a good tool as it is controlled to study the actions of a single viral protein's actions in the CNS in an inducible manner (Chauhan et al., 2003). In the included studies, opioids' ability to modulate behavioral deficits was

examined. A number of motor behaviors were chosen to test, as the striatum is a target for HIV-1 and is a convergence point with the opioid system (Tempel and Zukin, 1987), which has a dense population of receptors in this brain region. The slow induction of Tat in our Tat-transgenic mouse line and the examination of motor behaviors used in HIV-1 patients, allows us to examine changes in a relevant manner. While few studies have been performed by our group using morphine to assess behavior, herein we hope to develop a model for future use to not only examine the deficits incurred by HIV-1, but the mechanisms involved.

## **Materials and Methods**

### **Animal Model**

As discussed in published works by our lab (Bruce-Keller et al., 2008;Fitting et al., 2010a;Hauser et al., 2009), mice expressing the *tat* gene under the control of a *tet* responsive element (TRE) in the pTREX vector (Clontech, Mountain View, CA) were crossed with mice expressing the reverse tetracycline transactivator (RTTA) driven by a human GFAP promoter. Therefore, inducible Tat<sup>+</sup> transgenic mice express GFAP-RTTA and TRE-Tat genes, while Tat<sup>-</sup> mice only express the GFAP-RTTA gene. Mouse genotypes were determined by PCR analysis of tail clips. Tails clips were taken and digested in a chaotropic agent overnight at 37°C. DNA was extracted from the degraded tissue using the GeneElute™ Mammalian Total DNA kit (Sigma-Aldrich) according to the manufacturer's instructions and the purified DNA was then used in PCR reactions to amplify the starting material. PCR was performed to detect both the presence of the Tat

gene as well as the RTTA promoter using the following primers used previously (Bruce-Keller et al., 2008; Hauser et al., 2009): Tat forward: 5'-ATGGAGCCAGTAGATCCTAG-3'; Tat reverse: 5'-TCATTGCTTTGATAGAGAACTTG-3'; rTta forward: 5'-AATCGAAGGTTTAACCCG-3'; rTta reverse: 5'-TTGATCTTCCAATACGCAACC-3' (Chauhan et al., 2003). PCR products were separated by gel electrophoresis using a 1.5% agarose gel, were stained with ethidium bromide to visualize the DNA, and were imaged using a Kodak 440CF Image Station (Rochester, NY). Based on studies performed by, male mice are more susceptible to the behavioral deficits of Tat-induction than female mice. Therefore, only male mice were used in these studies. To understand the sex effects of HIV-1 Tat induction, refer to (Hahn et al., 2013).

### **Rodent treatment: Tat induction and morphine administration**

#### **Tat-induction**

Adult Tat<sup>+</sup> and Tat<sup>-</sup> mice, 2-3 months of age were given (during certain experiments) either normal chow (doxycycline control) or (during all experiments) chow containing doxycycline (DOX; 6 mg/g food; Harlan Laboratories, Inc., Indianapolis, IN) to induce the Tat transgene. The duration of *tat*-induction was either 1 month or 3 months depending on the experiment.

#### **Morphine administration**

In experiments involving morphine, the drug was administered either via subcutaneous injection or by a combination of subcutaneous pellet implantation and subcutaneous injection with morphine or, in the controls, implantation of placebo pellets

and injection of saline. In the former protocol, mice were injected twice daily at 12 h intervals with 20 mg/kg morphine sulfate. The latter paradigm is as follows:

	<b>Morning</b>	<b>Evening</b>
<b>Day 0</b>	<b>pellet implant</b>	<b>Mor 10 mg/kg s.c.</b>
<b>Day 1</b>	<b>Mor 20 mg/kg s.c.</b>	<b>Mor 20 mg/kg s.c.</b>
<b>Day 2</b>	<b>Mor 20 mg/kg s.c.</b>	<b>Mor 20 mg/kg s.c.</b>
<b>Day 3</b>	<b>Mor 40 mg/kg s.c.</b>	<b>Mor 40 mg/kg s.c.</b>
<b>Day 4</b>	<b>Mor 40 mg/kg s.c.</b>	<b>Mor 40 mg/kg s.c.</b>
<b>Day 5</b>	<b>Mor 80 mg/kg s.c.</b>	<b>Mor 80 mg/kg s.c.</b>
<b>Day 6</b>	<b>Mor 100 mg/kg s.c.</b>	<b>Mor 100 mg/kg s.c.</b>
<b>Day 7</b>	<b>Test</b>	

### **Gait analysis**

Animals were habituated to the testing environment for at least one hour prior to the procedure. Habituation to the gait analysis procedure occurred 4 days prior to any surgery or treatment in order to minimize any anxiety associated with the procedure. During the habituation, mice were restrained gently by neck scruffing on a wire cage top. Both front and back paws were dipped into non-toxic paint (Crayola) and mice were placed at the entrance of the gait analysis apparatus, a plexiglass corridor 75 × 6 × 10 cm with opaque walls and a clear ceiling and with an enclosed cardboard box at the end to provide a feeling of security and an impetus to traverse the apparatus. Mice were released from restraint and allowed to explore and pass through the apparatus. Habituation was performed once per day for 3 days with the 4<sup>th</sup> day serving as the baseline. During testing, mice were scruffed, and front paws were dipped in blue paint, while back paws were dipped in red paint. The gait analysis corridor was lined with absorbent white receipt paper and mice were allowed to traverse the apparatus. Mice were immediately removed from the dark box at the end and put in an empty cage to allow the paint to be removed from their skin/fur before returning to their home cage

containing bedding. Footprint-containing papers were removed from under the apparatus and hang-dried. Measurements of stride-length, sway width, and stance length were all taken from dried forepaw prints. No differences were noted in gait measures between measures made with forepaws and those made with hindpaws (unincluded observations).

### **Locomotor activity**

#### **One time, unhabituated, novelty test**

Mice were habituated to the testing room for at least one hour prior to testing. Locomotor activity was assessed in clean activity chambers (30 × 30 × 15 cm) with cameras mounted overhead with ANYMAZE software which tracked the movement of animals over a 10 min period and a freeze cut-off time of 3 s. Activity counts were converted to total distance traveled for comparison between groups.

### **Forelimb grip strength test**

Mice were habituated to the testing environment for at least one hour prior to performance of the test, at which point animal weights were taken. Grip strength was measured using a push-pull strain digital force gauge, Chatillon® DFE II grip strength meter (AMETEK's Test & Calibration Instruments (TCI), Largo, Florida). The meter was attached to a forelimb grip pedestal composed of a wire grid, similar to a cage top, to which the mice were able to grab ahold. Mice were held gently by the base of the tail and lowered down to the pedestal until they took hold of the grid. Mice were then pulled back gently from the pedestal until it was released. Peak force was automatically

measured in grams-force (gf) by the apparatus (Grip Strength Meter 2.66, AMETEK's Test & Calibration Instruments (TCI)) and recorded by the program. Mice performed 5 trials in quick succession and the greatest measurement was used for comparison to the other test subjects (Crabbe et al., 2003). Measurements of grip strength (g) were normalized to body weight (g) to examine differences between groups.

### **Warm water tail-immersion test**

Prior to assessment, mice were acclimated to the testing conditions for at least one hour. The warm water tail-flick test was performed according to (Coderre and Rollman, 1983) using a water bath with the temperature maintained at  $56 \pm 0.1^{\circ}\text{C}$ . A baseline (control) tail-flick latency was taken prior to morphine injection and/or pellet implantation and were performed on each test day. Following treatment of either morphine or saline (injection) or morphine and placebo (pellet), tail-flick latencies were measured 30 min after injections were given. Mice were covered and 2/3 of their tails were submerged into the warm water. The latency to flick or lift their tails out of the water was measured, with a safety cut-off time of 10 seconds in place to prevent tissue damage from occurring. Antinociception was converted to percent maximum possible effect (%MPE) according to (HARRIS and PIERSON, 1964) which was calculated according to the following formula  $\%MPE = [(test\ latency - control\ latency) / (10 - control\ latency)] \times 100$  (Hull et al., 2013).

### **Pilot study for changes in gait behavior**



Tat<sup>-</sup> and Tat<sup>+</sup> mice were treated with DOX chow for 3 months. Mice were habituated to the gait analysis apparatus over three trials performed on the day of the test, with the fourth pass through the apparatus used as the measure of gait analysis. Habituation was performed because the restraint required to adequately paint the mouse paws had the potential to cause anxiety-like behavior which could confound the gait analysis results, as the speed with which the rodents pass through the apparatus can affect the gait measurements.

#### **One month DOX induction with a 2-week twice-daily morphine injection**

Because other measures of motor behavior were able to show significant differences within shorter treatment periods using the rotarod (Fitting et al., 2012), a shortened DOX treatment schedule was employed. Tat<sup>-</sup> and Tat<sup>+</sup> mice were treated with DOX for two weeks. During the last three days of the two weeks, mice were habituated to the gait analysis procedure, and on Day 15 pre-opioid administration, gait analysis and grip strength measures were taken. Mice continued to consume DOX chow for another two weeks, during which time they were injected twice daily at 7:00 am and 7:00 pm with 20 mg/kg morphine sulfate or saline control, subcutaneously. Following two-weeks of morphine administration, mice were tested in a number of parameters: locomotor activity, grip strength, gait analysis, and tail-flick.

#### **Three-month DOX treatment with 75 mg morphine pellet implantation and ramping morphine injections**

Because of the effects that were seen with locomotor activity in Tat<sup>+</sup> mice administered morphine, in addition to the lack of any changes in motor behavior after only one month, the protocol was adjusted to a longer time point (see Results section). Additionally, to attempt to identify the source of the genotypic variation in the response to morphine, mice were administered 75 mg morphine pellets and ramping injections of morphine to induce tolerance and allow us to manipulate the challenge doses of morphine in such a way as to determine whether effects were due to differences in the efficacy of morphine between the genotypes or the Tat transgene itself. Before morphine or placebo pellets were implanted, mice were habituated to the locomotor activity chamber for three days for 30 min each, and the fourth day the activity counts were used as the baseline; the same was done for the gait analysis apparatus. Pellets were then implanted on day 0 and the above protocol followed for morphine injection. Locomotor activity was assessed daily along with gait analysis, with tail-flicks performed every 2 days. Grip strength was only performed on test day (day 7) because novelty was found to be important in this assay.

## **Results**

### **Pilot study for doxycycline-induced motor impairments**

Gait analysis is a task that had not been previously performed by members of our laboratory, though past paradigms were used as a guide (Girirajan et al., 2008). Initially, for the first studies, mice were habituated to the conditions of the assessment within one testing period, i.e. they were given three habituation trials and the fourth was the one

recorded for comparison to other groups. In this study, no non-doxycycline chow eating controls were used; both the Tat<sup>-</sup> and Tat<sup>+</sup> mice consumed doxycycline rodent chow for a period of three months. Utilizing this method, a significant difference in the gait parameters of stride length and sway width were found between Tat<sup>-</sup> and Tat<sup>+</sup> mice, with Tat<sup>+</sup> mice displaying both longer stride length and wider sway width (Figure 4.1).

### **One month doxycycline induction with two-week morphine administration regimen**

Tat<sup>-</sup> and Tat<sup>+</sup> mice were treated with DOX for 2 weeks prior to beginning a twice daily subcutaneous (s.c.) injection of saline or 20 mg/kg morphine. After 2 weeks of DOX treatment, no changes were seen in grip strength (Figure 4.3A) or gait analysis (Figure 4.4A) between Tat<sup>-</sup> and Tat<sup>+</sup> mice, suggesting that this briefer period of DOX induction was not sufficient to cause motor impairments using these parameters. Additionally, another two weeks of DOX induction did not result in motor impairment in either measure of grip strength (Figure 4.3B) or gait parameters (Figure 4.4B), as compared to saline groups either. Interestingly, in this situation, treatment with morphine had significant effects on both measures (Figures 4.3B, 4.4B). Morphine treatment caused a significant increase in the gait parameters of stride length and stance length but only in the Tat<sup>-</sup> mice. Two-way ANOVA with Tukey post hoc. \*\*  $p < 0.005$  vs. saline control,  $n = 7-8$ . However, there was no difference between the Tat<sup>-</sup> group and the Tat<sup>+</sup> group on morphine. Additionally, mice, regardless of genotype, had greater grip strengths following the morphine treatment paradigm than their saline treated counterparts. Two-way ANOVA with Tukey post hoc. \*\*  $p < 0.005$  vs. saline control,  $n = 7-8$ . Locomotor activity, which was examined for a 10 minute period in activity

chambers, revealed a significant increase in the locomotor activity of Tat<sup>-</sup> mice administered morphine (Figure 4.5). Elevation in locomotor activity in response to opioid administration is a well-described phenotype. However, interestingly, this phenotype was not present in mice possessing the Tat transgene; their locomotor activities mirrored those of their saline counterparts. Two-way ANOVA with Tukey post hoc analysis revealed an interaction of genotype × treatment. \*\*\* p < 0.0001 vs. saline control, n = 7-8.

#### **Long-term doxycycline induction with 75 mg morphine pellet and ramping morphine protocol**

A pilot study was performed with the help of David Stevens of the Dewey laboratory to examine the antinociceptive effects of 75 mg morphine pellets ± ramping morphine injections on the development of tolerance between Tat<sup>-</sup> and Tat<sup>+</sup> mice. Pellet implantation alone produced tolerance, which decreased with increasing morphine challenge doses, however there was no difference in antinociceptive efficacy between genotypes (Figure 4.6A). However, when ramping morphine injections were performed in addition to implantation of pellets, there was a divergence of the responses, where Tat<sup>-</sup> mice showed a greater antinociceptive response to morphine challenge at 32 and 64 mg/kg than did the Tat<sup>+</sup> mice, while challenges at both lower doses (16 mg/kg) and higher doses (128, 256, and 512 mg/kg) elicited similar responses (Figure 4.6B). This was a pilot study performed on very few mice, n = 2, thus differences cannot be determined to be statistically significant. However, it was thought possible that when using the 75 mg pellet protocol combined with ramping morphine injections, that on test

day, challenging animals with either 16 mg/kg or 64 mg/kg and examining their responses could reveal not only differences between genotypes, but the mechanism of these differences.

During habituation of mice to the locomotor activity boxes, there was no initial difference in distance traveled, as there was a lot of variability among subjects. At Day 4 of habituation, when baseline locomotor activity was taken, there were no differences among genotype and counts had reduced to approximately half of their Day 1 numbers. Following morphine pellet implantation and ramping morphine injections, significant differences in activity were seen. Differences became apparent at Day 2 and remained throughout the entire time that the pellet was on board, however, they were not genotype-specific. Tat<sup>-</sup> and Tat<sup>+</sup> mice consuming normal rodent chow both showed significant locomotor activation in response to morphine, which is expected as morphine is well known to elevate locomotor activity in response to morphine (Carroll and Sharp, 1972), and evidence suggests that it is correlated to striatal dopamine levels (Saito, 1990). However, what was not anticipated, was that the ablation of the locomotor activating effect would be observed in both types of mice given DOX chow, suggesting that the DOX treatment over a long period of time, and combined with this morphine treatment were having effects on their own. Because of the strength of the morphine treatment, an effect of drug is observed between the vehicle and morphine groups, with the morphine-treated mice having significantly lower body weights.

## **Discussion**

The use of cART therapy has been instrumental in allowing the HIV-1 infected population to survive into their second decade. However, these drugs have been ineffective at combating neurocognitive disorders, though they are able to shift the spectrum of the disorders towards the less severe (Heaton et al., 2011; Joska et al., 2010). This, with the knowledge that symptoms of HAND are present in the absence of virus in the brain, suggests that the CNS is uniquely vulnerable to this disease (Medigue et al., 1990; Le, V et al., 2012). As 50-60% of those infected with HIV-1 experience HAND to some degree (Ellis et al., 2007), it is important to understand the mechanisms by which these symptoms are resistant to cART treatment. Although cART drugs are notorious for their inability to cross the BBB and their additional toxicity on their own (Letendre et al., 2008; Antinori et al., 2005; Nau et al., 2010), the presence of impairments in the absence of virus suggests that specific cART cocktails have improved to a level in which they are crossing the BBB, but are just not effective at preventing the CNS damaging effects of HIV-1 viral proteins. Being able to mimic the types of disorders that are experienced by HIV-1 patients using an animal model is important to the study of the underlying causes of these impairments as well as ways in which to target them. The Tat-transgenic mouse model is a good tool with which to study the chronic effects to HIV-1, because experimenters can isolate the effects of HIV-1 proteins in the CNS, and have the ability to manipulate the length of time that the animal is exposed to the HIV-1 protein. Because little protein is made in comparison to other models, a broad spectrum of effects, both short-term and long-term, can be examined (Kim et al., 2003).

In addition to assessment of HIV-1 protein effects, the in vivo effects of opioids in combination with HIV-1 are also of interest. The majority of the work in the lab models HIV-1 and opioid interactions by using cell culture techniques, however, because cell culture removes the cells from their normal connections, it is important to confirm our findings using these types of models which also allows us to be able to extend our studies to mimic symptoms, testing paradigms, and treatments to those that are seen and/or used with humans. Additionally, to achieve cell cultures that are selective for specific cell types and that will survive and propagate well in culture, neonatal (P0-1) pups and embryonic (E15-16) animals are used, which lack some of the same processes and immune functions that their adult counterparts have developed (Mestas and Hughes, 2004;Holsapple et al., 2003).

Chronic opioid treatment has been shown to lead to glial activation (Mattioli et al., 2010;Raghavendra et al., 2004;Hutchinson et al., 2007;Watkins et al., 2007b;Watkins et al., 2007a;Watkins et al., 2005) using a number of treatment paradigms. Glial activation exerts control over morphine's response, leading to the promotion of tolerance, exacerbation of withdrawal symptoms and promotion of rewarding effects (Narita et al., 2006;Eidson and Murphy, 2013). In a number of cases, these effects can be reversed by applying low dose opioid antagonists, or by using agents purported to selectively reduce glial activation such as profentolymine, minocycline, and ibudilast (Raghavendra et al., 2004;Mattioli et al., 2010;Bland et al., 2009;Lee et al., 2009). Changes to morphine's effectiveness as a therapeutic or changes to its rewarding properties though direct actions or indirect actions, such as through the development of tolerance could influence morphine's utility as a therapeutic agent. Additionally,

morphine's rewarding/antinociceptive properties can alter the drug intake level in those abusing the substance because increased drug may be required to combat the development of tolerance. An additional reason for studying the chronic effects of opioids in these models instead of only single injections, though the beginnings of the cascades leading to glial activation, tolerance, withdrawal, etc. stem from these first injections, is that it's a way in which to separate morphine's analgesic effects from its inflammatory-inducing role, which seems to be affected temporally as well as by the quantity ingested. As morphine is known acutely to both block pain and to be an anti-inflammatory agent (Pourpak et al., 2004), a more acute morphine regimen will likely give opposing results.

The treatment paradigms that were selected, which involved either morphine injections or morphine pellet implantation and injections, were done so as to be able to mimic the highs and lows of morphine intake similar to what occurs in those using the drugs by injection. These may even be more pronounced in rodents, as the half-life of morphine is much shorter in mice (~42 minutes) than in humans (2-4 h) in mouse whole blood (Ishikawa et al., 1982).

The first study did not involve morphine drug administration and was used to assess the ability of long-term Tat induction to alter basic motor behaviors, which it was able to do in measures of stride length and sway distance after a 3-month induction. Gait analysis is a task performed in many test batteries for neurocognitive impairment in HIV-1 patients, and can include a natural gait test or assessment of timed fast-walk (Robertson et al., 2006; Stern et al., 2001). The increase in sway distance was expected in the Tat<sup>+</sup> animals compared to the Tat<sup>-</sup> animals, because this suggests a less stable



gait. However, the difference in stride length between the genotypes was not expected, as, if we predicted a more unstable gait, would result in a shortened stride. We observed that the  $Tat^{+}$  mice seemed more anxious during handling. To address this concern, we decided to prolong the habituation period of the animals in the future. Another study performed in our laboratory determined that male  $Tat^{+}$  mice spent significantly less time in the light side of the light-dark box than male  $Tat^{-}$  mice (Hahn et al., 2013), suggesting that anxiety-like behavior might confound the gait analysis procedure.

Due to studies performed in the lab (Fitting et al., 2012) that identified a reduction in rotarod performance following three weeks of DOX exposure, it was decided to employ a shorter DOX induction protocol to determine if we could recapitulate those more rapid deficits in our other models of motor impairment. Unfortunately, neither two nor four-weeks of DOX induction were able to produce changes in gait performance or grip strength between  $Tat^{-}$  and  $Tat^{+}$  animals. The reason for this may be due to the differences in difficulty between the behaviors. The more difficult the task, the easier it is to reveal impairments, while more moderate tasks may take longer to differentiate between the impaired and unimpaired. Additionally, acute morphine injections after two weeks of DOX induction produced no effect on grip strength or gait analysis behaviors. In these behaviors, however, after chronic morphine treatment, differences were noted in both grip strength and gait analysis, but in ways that were not expected for these types of tests. We expected, after chronic morphine treatment, that motor behaviors would be impaired, i.e. that decreases in grip strength and shortened stride length and sway distances would be observed. However, both an increase in grip strength in both

the Tat<sup>-</sup> and Tat<sup>+</sup> animals and an increase in stride length and stance length in the Tat<sup>-</sup> animals were noted. Morphine is known to have a locomotor activating effect which correlates to dopamine release in the striatum (Murphy et al., 2001; Vihavainen et al., 2006). Morphine, after the two weeks of treatment, still retains most of its efficacy in the tail-flick, suggests that locomotor activation could account for the change in both stride length and stance length (which is influenced by the stance length) that were seen after chronic morphine. Interestingly, this effect did not occur in the Tat<sup>+</sup> animals, and while the Tat<sup>-</sup> and Tat<sup>+</sup> animals were no different from one another statistically, the Tat<sup>+</sup> stride length and stance length were not different from control as they were with the Tat<sup>-</sup> mice. This could be due to the decrease in morphine efficacy in Tat<sup>+</sup> animals after two weeks compared to an acute dose of morphine at this time. And while there was no difference in the Tat<sup>-</sup> and Tat<sup>+</sup> tail-flick responses, there was a trend. To confirm the reason for these effects, more work needs to be done using a range of doses of morphine to test the relationship between morphine efficacy and effects on gait analysis. It is interesting that an acute dose of morphine did not produce an increase in stride length and stance length, and could suggest that sensitization occurs to this type of behavior (Valjent et al., 2010; Becker et al., 2001). During the grip strength behavior, mice treated with chronic morphine appeared to be more inclined to grab onto things such as the experimenter's fingers/hand as well as the grid used for the grip strength assessment. Thus, the mouse's desire to affix itself to something skews the results of the test and as in the case of the gait analysis examination, a range of morphine doses need to be tested in order to paint a more complete picture of these effects. Additionally employing a morphine treatment paradigm in which tolerance develops would, most likely, allow us

to see a different profile for morphine's effects. However, though we were disappointed with a number of behavioral effects in which morphine did not show genotypic differences, we were excited to find that one motor behavior did indeed respond in, what we think is an interpretable manner. We found that in a novel 10 min. session in a locomotor activity chamber, that Tat<sup>+</sup> mice did not have the locomotor activating response to morphine that the Tat<sup>-</sup> mice displayed, which suggests that there could be a genotypic difference in morphine efficacy that is accounting for this effect or that the presence of the tat-transgene is altering a process important for locomotor activation. While this is interesting, another Tat-transgenic mouse model created by Johnny He (Kim et al., 2003) has shown unpublished data that morphine treatment along with Tat-induction actually increases locomotor effects (McLaughlin et al., unpublished), though this lab has published similar effects with Tat-transgenic animals and the effects of cocaine administration (Paris et al., 2013). The tat-transgenic mouse line is a very useful approach, but doesn't allow us to target specific brain regions. Histology on multiple brain regions such as the striatum, cerebellum, etc. should be assessed for changes to neuron arborization and for glial activation to assess physiological changes to the CNS and would allow assessment for the potential of regional-specific effects. Additional experiments could include targeted injections of Tat into the striatum to attempt to recapitulate the behavioral changes found using this approach.

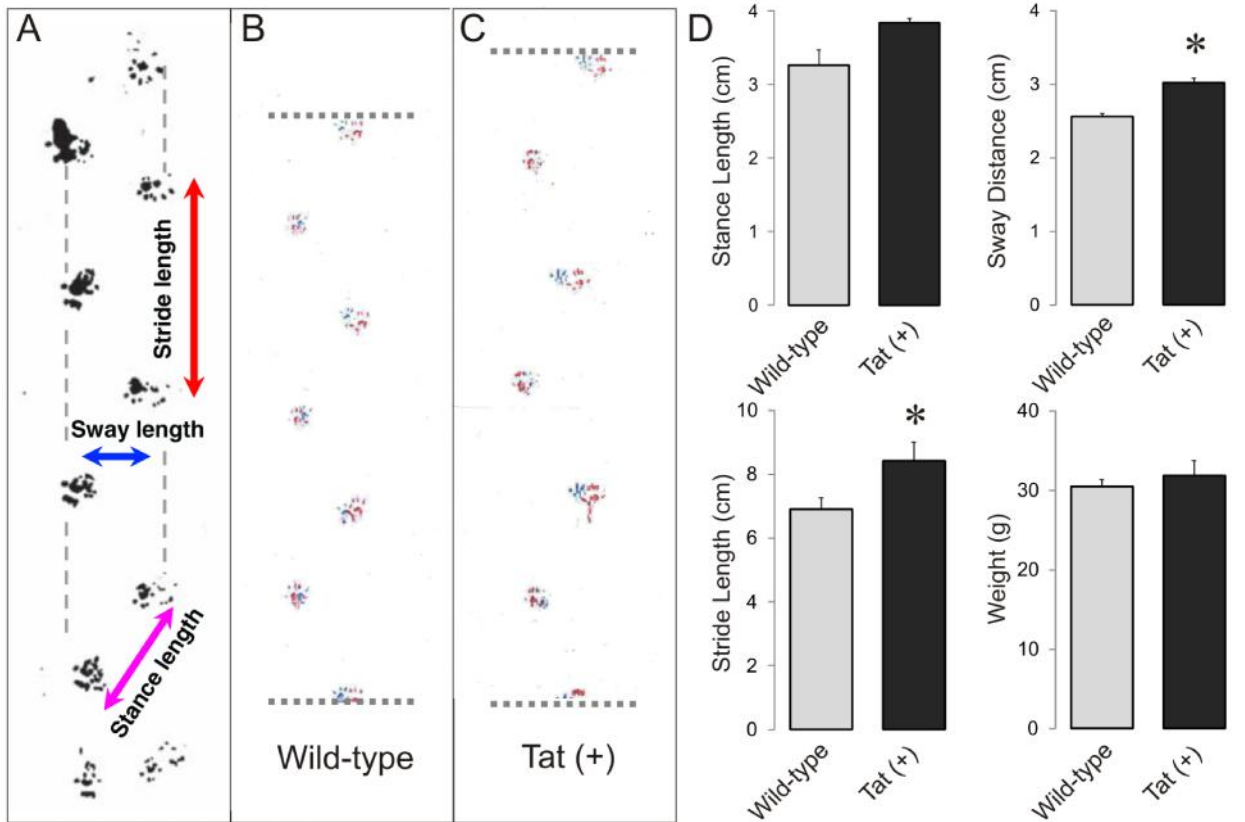
For the above reasons concerning the lack of difference in motor behaviors between Tat<sup>-</sup> and Tat<sup>+</sup> after one month of DOX treatment, differences in morphine efficacy between the Tat<sup>-</sup> and Tat<sup>+</sup> and the lack of change in the majority of the behavioral effects in the motor behavioral assessments, the protocol was adjusted both

to a time in which differences in motor behavior were previously noted, and a morphine treatment paradigm was employed in which tolerance has been shown to develop. Unfortunately, long-term DOX treatment had side effects that interacted with morphine treatment. Long-term DOX treatment for three months produced a non-genotype specific effect when long-term DOX treatment was combined with the morphine treatment. Whether or not this was a specific effect in response to the degree of morphine application or whether this effect would have been displayed in response to more moderate morphine injection is unknown. However, data from other laboratories (Miller, unpublished) suggests that in a model of ICSS, doxycycline effects are noted in response to inter-cranial self-stimulation, which is not a drug treatment, but activates similar pathways as morphine and other drugs of abuse (Carlezon, Jr. and Chartoff, 2007; Vlachou and Markou, 2010). Because of these off-target effects, the interpretation of these results is not possible, but suggests a weakness in the model. Because of these effects, it might be best to perform tests examining only the effects of Tat, or exploit the effects of tat-induction with morphine administration using tests that show differences in the more short-term, such as rotarod performance and locomotor activity. Additionally, the morphine pellet protocol should be performed to determine if the strength of the morphine treatment will produce off-target effects even at times such as one-month of *tat*-induction, i.e. if the strength of the morphine treatment is either creating or exacerbating this effect.

Overall, these behavioral studies suggest that motor behaviors are affected by induction of the *tat*-transgene, while morphine is able to modify some of these responses. However, more studies need to be done to determine the best methods to

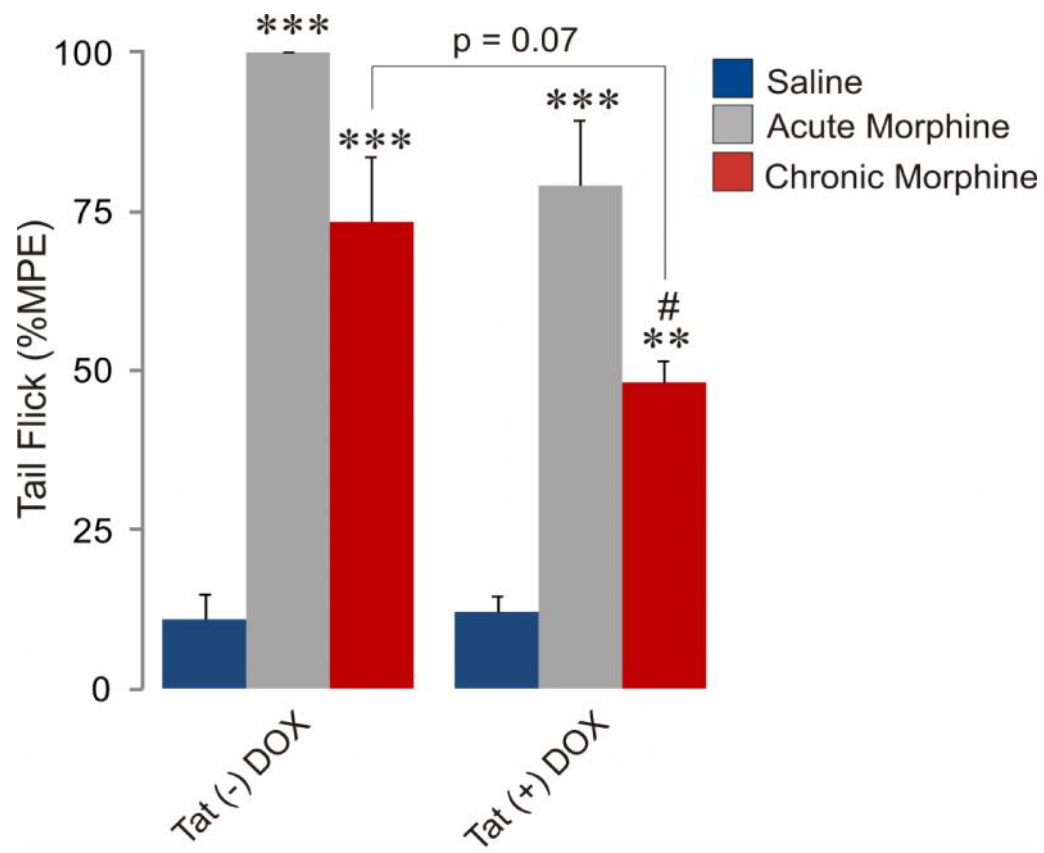
combine morphine administration with *tat*-induction to be able to effectively determine the interactions of these two exogenous substances.

Figure 4.1



**Figure 4.1** (A) Stride length (red arrow), sway width (blue arrow) and stance length (pink arrow) were used as measures of gait in our studies. Measurements were taken between forepaws on dried papers that had been laid out under the gait apparatus and walked upon as the mouse traversed the gait box. Panel A was modified from (Girirajan et al., 2008). (B) and (C) Example paw print images of  $Tat^{-}$  (B) and  $Tat^{+}$  (C) gait performance. Forepaws were dipped in blue paint, while hindpaws were dipped in red paint to make them easy to distinguish on the papers. Gait parameters were measured as shown in A). While changes in the sway distance and stance length are more difficult to note overtly, it is easy to see the difference in stride length between the two genotypes. D)  $Tat^{-}$  and  $Tat^{+}$  mice treated with DOX for three months showed no differences in stance length, but both the stride length and the sway distance of the  $Tat^{+}$  animals was greater than the  $Tat^{-}$  animals. \*  $p < 0.05$  vs.  $Tat^{-}$ , results of unpaired t-test,  $n = 3 - 4$ .

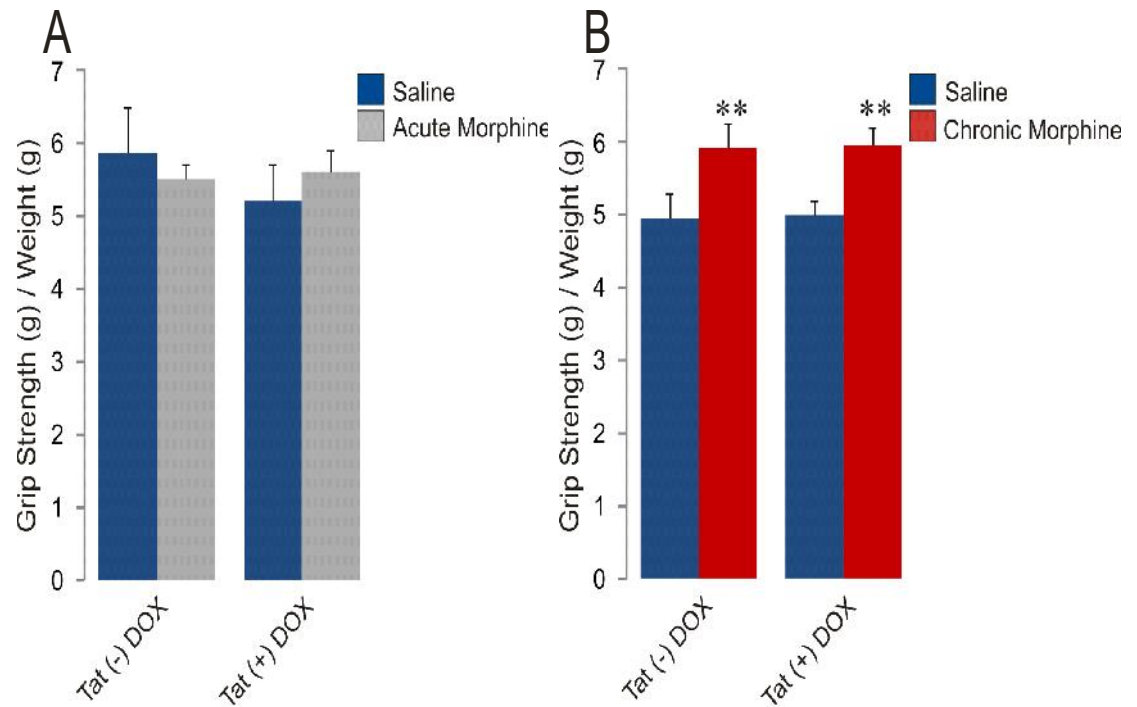
**Figure 4.2**





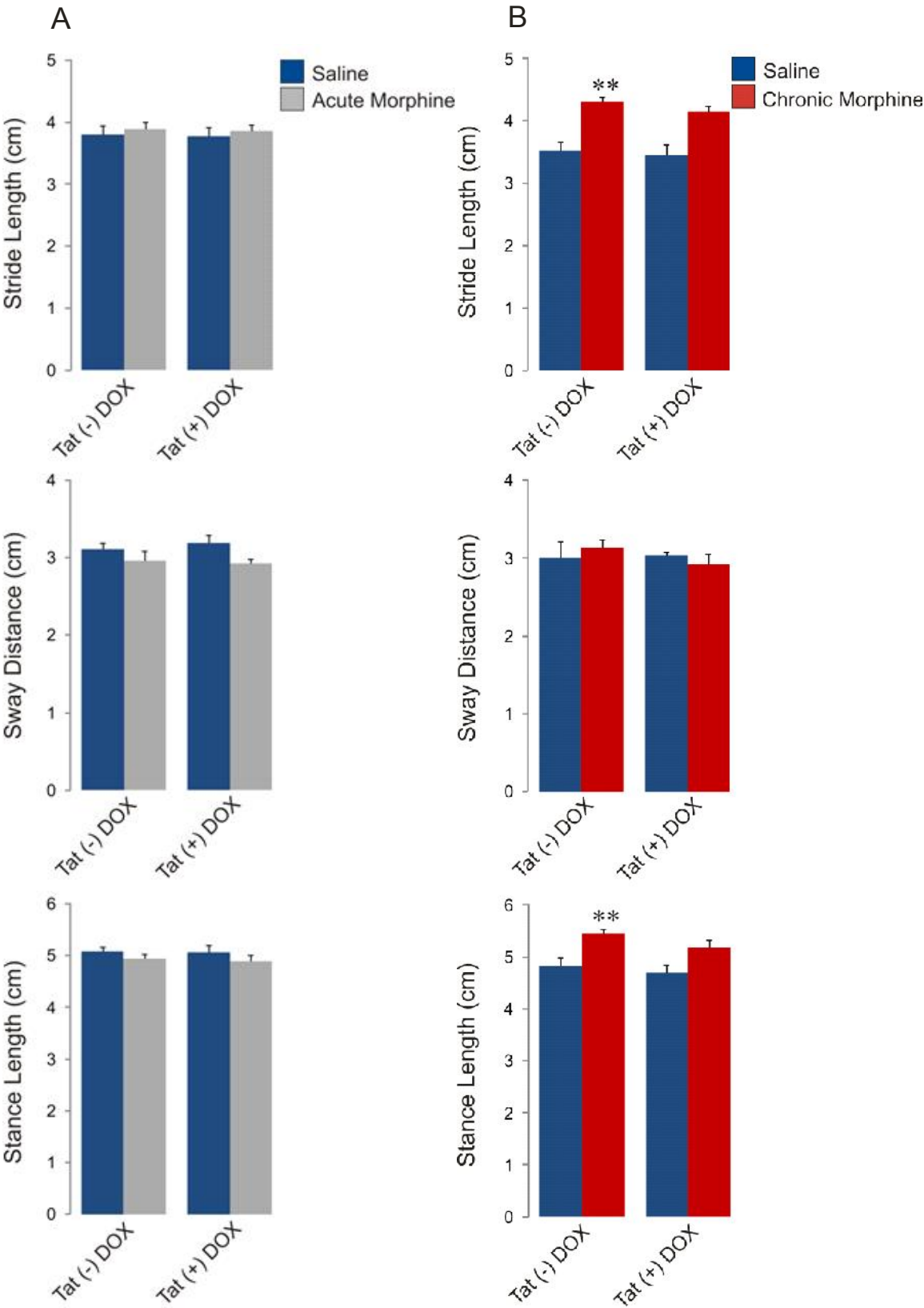
**Figure 4.2** Antinociceptive effect of 20 mg/kg morphine following either a single injection (A) or two-weeks of twice-daily administration of the drug (B). In Tat<sup>-</sup> mice, saline controls elicited a rapid tail-flick response, while a single injection of 20 mg/kg morphine was able to produce a full antinociceptive effect in the tail-immersion test, \*\*\*  $p < 0.0001$  vs. saline control. Following two weeks of twice-daily injections, the 20 mg/kg challenge dose continued to produce ~75% effect, which was not statistically different from the effect of the acute dose, \*\*\*  $p < 0.0001$  vs. saline control. In the Tat<sup>+</sup> mice, saline produced a similar response across genotypes. The acute dose of morphine produced an ~75% effect, \*\*\*  $p < 0.0001$  vs. saline control, while the challenge dose after chronic morphine treatment produced an ~50% effect. A significant difference was found between the acute and chronic morphine treatments in the Tat<sup>+</sup> mice, #  $p < 0.05$  vs. acute morphine, and a trend,  $p = 0.07$  between the two chronic morphine groups.

**Figure 4.3**



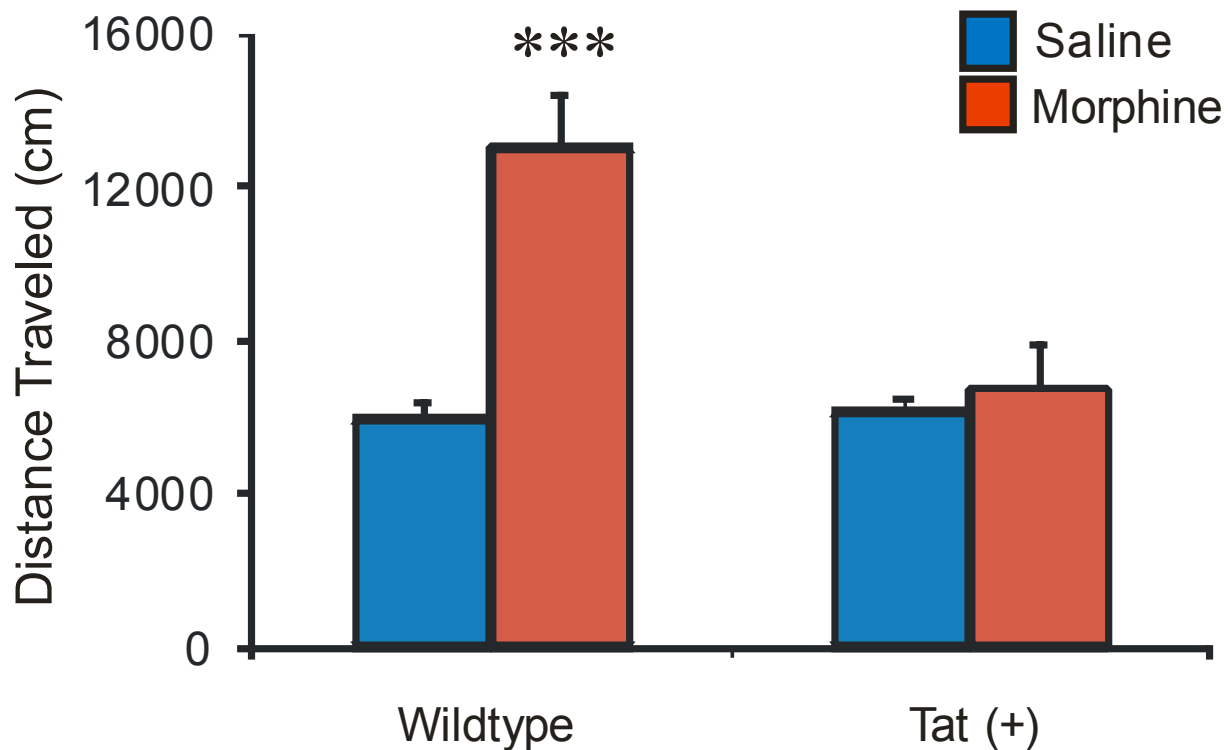
**Figure 4.3** Grip strength assessments in mice following saline (A) & (B), a single injection of morphine (A), or two weeks of twice-daily morphine injections (B). Grip strength measures in grams of force were normalized to the body weight of each animal to yield a unit-less value. There was no difference between Tat<sup>-</sup> and Tat<sup>+</sup> mouse grip strength values after two weeks or one month treatment with DOX, and an acute morphine injection had no effect on the behavior. However, in both genotypes of mice, chronic morphine injections caused an increase in the grip strength of the animals. \*\* p < 0.005 vs. saline control, n = 7 - 8.

Figure 4.4



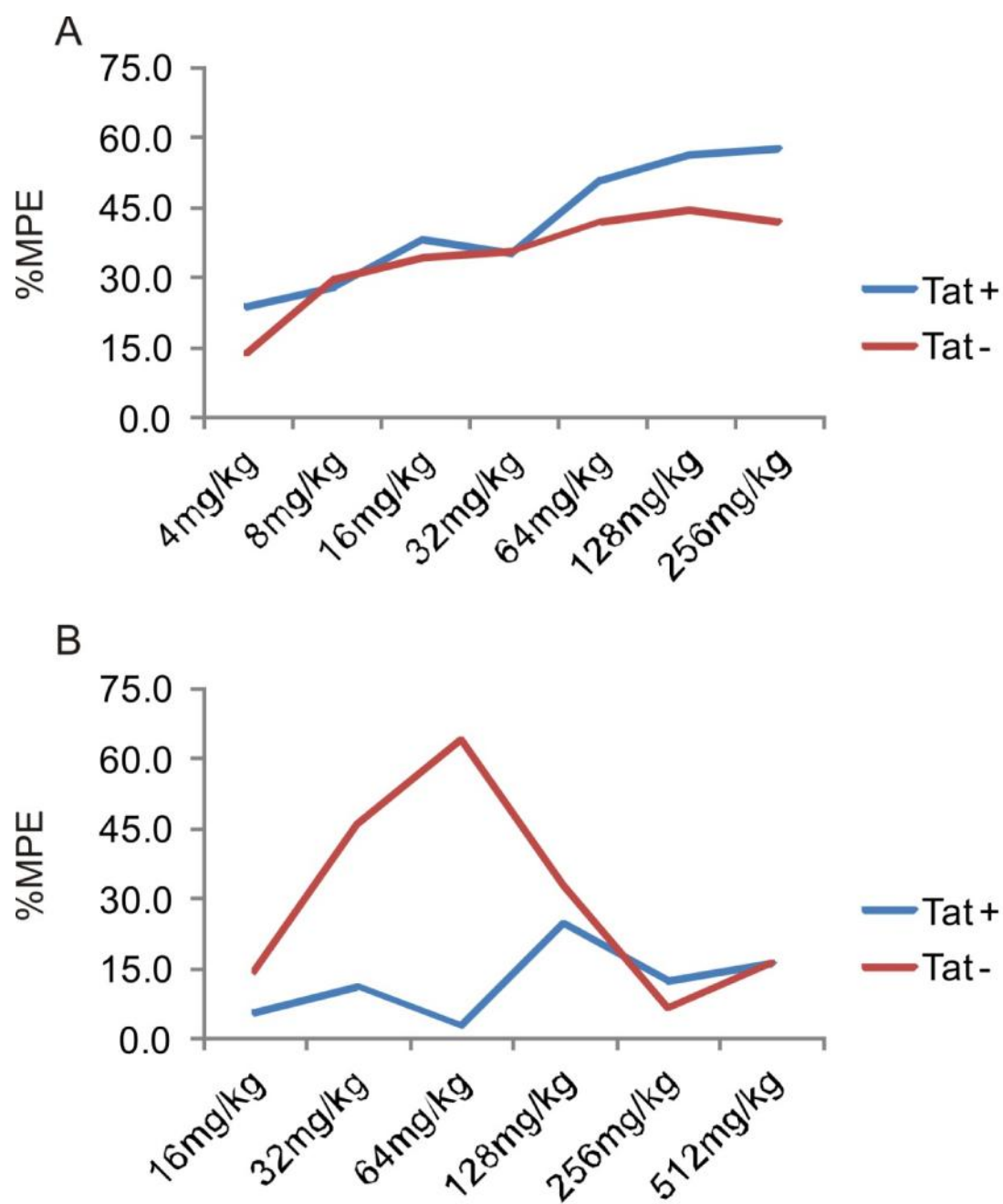
**Figure 4.4** Gait analysis performed following two-weeks and four weeks of DOX induction with and without morphine administration. (A) Two week induction of Tat did not result in any changes in gait analysis performance in any of the parameters tested between the Tat<sup>-</sup> mice and the Tat<sup>+</sup> mice, nor did a single injection of morphine at 20 mg/kg in any alterations in these measures. (B) An additional two-weeks of DOX treatment was not sufficient to result in changes in gait in the control, saline treated animals. However, mice administered morphine chronically did show changes to their gait behavior. Significant increases in stride length and stance length were noted in those animals treated with morphine for two-weeks. \*\*  $p < 0.005$  vs. saline control,  $n = 7-8$ .

**Figure 4.5**



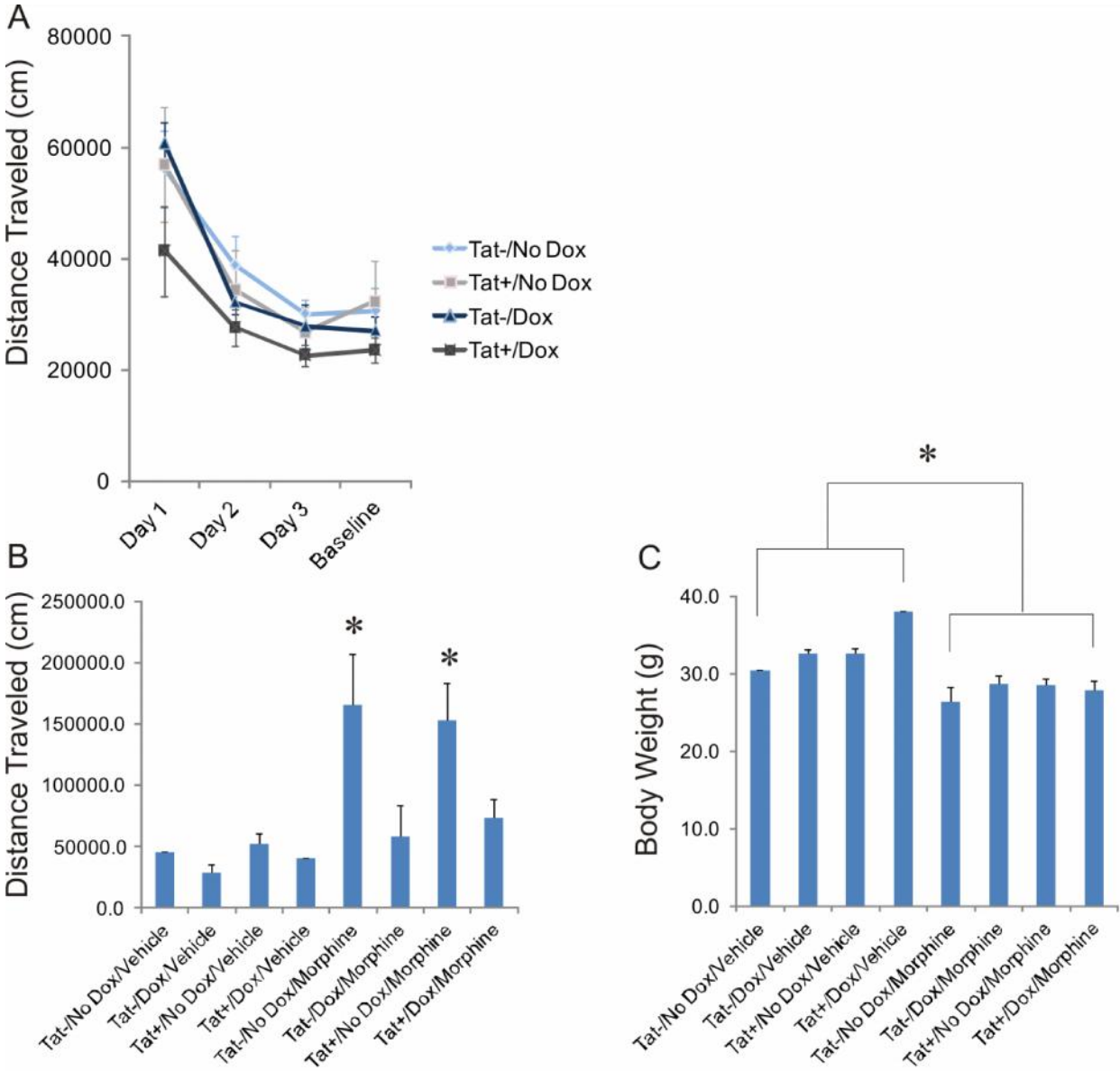
**Figure 4.5** Locomotor activity measures performed in Tat<sup>-</sup> and Tat<sup>+</sup> mice treated with saline and morphine. Animals performed this task only once for a period of 10 minutes and the bodies of the animals were tracked using ANYMAZE software. Locomotor activity did not differ between the Tat<sup>-</sup> and Tat<sup>+</sup> mice administered saline, suggesting that there was no change in the genotypic response on its own. Tat<sup>-</sup> mice administered morphine for two-weeks exhibited the classic elevation in locomotor activity behavior, \*\*\*  $p < 0.0001$  vs. saline control however, this response was not observed in the Tat<sup>+</sup> mice.

Figure 4.6



**Figure 4.6** Pilot study of tail-flick responses in Tat<sup>-</sup> and Tat<sup>+</sup> mice administered either 75 mg morphine pellets alone (A) or 75 mg morphine pellets in combination with ramping morphine injections for one week and then challenged with increasing doses of morphine (B). (A) Surgical implantation of 75 mg subcutaneous morphine pellets showed little difference between Tat<sup>-</sup> and Tat<sup>+</sup> mice in their response to morphine challenge doses. However, when mice were implanted with 75 mg morphine pellets and given ramping doses of morphine (B), there was a separation in the antinociceptive actions of the challenge doses between genotypes. At 32 mg/kg and 64 mg/kg the antinociceptive effect of morphine was greater in Tat<sup>-</sup> mice compared to Tat<sup>+</sup> mice. However at the lower and higher challenge doses, 16 mg/kg and 128, 256, and 512 mg/kg, respectively, no difference in effect between the genotypes was found. n = 2.

Figure 4.7





**Figure 4.7** Effects of long-term induction of DOX, morphine pellet implantation and ramping morphine injections on motor behaviors in Tat-transgenic mice. (A) After 3-months of DOX induction, mice were habituated to the locomotor activity chamber for 30 min each day for three consecutive days. On the fourth day, a baseline locomotor activity measure was taken, and no groups were statistically different from one other in their measures of distance traveled. (B) Representative graph of Day 4 of locomotor activity following morphine pellet implantation as days 2-6 showed the same pattern of response. Animals receiving placebo pellets and vehicle injections, whether or not they were fed DOX or normal chow showed measures of distance traveled similar to their baseline responses. Both Tat<sup>-</sup> and Tat<sup>+</sup> animals that were fed normal chow and treated with morphine showed locomotor activation by morphine, however, both the Tat<sup>-</sup> and Tat<sup>+</sup> animals that received the DOX chow and were given morphine injections failed to exhibit the locomotor activating effects of morphine. (C) Morphine pellet implantation and ramping injections caused a significant change in body weight compared to those mice receiving placebo pellets and vehicle injections. (\*  $p < 0.05$  vs. respective saline control, two-way ANOVA, Tukey's post hoc,  $n = 3 - 4$ ).

## Chapter 5

### Final Conclusions

The goal of these studies was to assess the utility of a CCR5 antagonist and cART therapeutic agent, maraviroc, as well as the CCR5 receptor, *per se*, in its involvement in opioid-HIV-1 interactive effects in cells of the striatum. These efforts, in an environment in which neurocognitive decline is prevalent, while mortality and viral replication are under control, are important to the understanding of inflammatory and neurodegenerative mechanisms that contribute to the disease spectrum, HAND. To this end, we examined the effects of two HIV-1 proteins with disparate interactions with the CCR5 receptor, gp120<sub>ADA</sub> and Tat<sub>1-86</sub>, and assessed their interactions with opioids in measures of neurotoxicity and/or neurodegeneration, glial inflammatory signaling, and the role of maraviroc in the interactive effects.

While gp120<sub>ADA</sub> did not interact with morphine in its neurotoxic actions and was not a worthwhile pursuit of study when interested in morphine and HIV-1 co-morbidity, the aberrant signaling responses that lead to neuron death are still of interest when studying the potential utility of maraviroc as a cART therapy. It will be important to show whether maraviroc can reverse actions of gp120<sub>ADA</sub> on its own, which as a CCR5 antagonist with a strong ability to block viral entry, it should be able to do. Thus, gp120<sub>ADA</sub>'s lack of interaction with morphine may be less important than its ability to be modified by drug treatments that may also have action at blocking morphine-HIV-1 interactive effects. While maraviroc failed to modify the effect of Tat on its own in my studies, CCR5 knockout in glia, or neurons and glia, but not in neurons alone

attenuated Tat-mediated neurotoxicity. Whether this disparity between pharmacological and genetic approaches is due to the low concentration of maraviroc used, its low potency in mice (Pfizer Inc., 2007), or an increased strength of conditional genetic manipulation is unclear. The maraviroc concentration was limited to 50 nM due to off-target effects reported above 100 nM at MOR in primary cells (Pfizer Inc., 2007), though studies in cell-lines have shown that 100 nM maraviroc effectively blocked viral entry with seemingly no off-target effects (El-Hage et al., 2013). Previously, when examining NF- $\kappa$ B activation in response to HIV-1 Tat, one of the initial studies performed in this project, concentrations of 50, 75, and 100 nM of maraviroc were all used to determine if concentration-dependent decreases in NF- $\kappa$ B activation by Tat would occur. While 100 nM maraviroc suppressed NF- $\kappa$ B p65 activation to a greater extent than 50 nM maraviroc, there was no significant difference between the concentrations of inhibitor tested. Additionally, time-lapse studies examining neuron mixed-glia co-cultures with 100 nM maraviroc produced significantly elevated toxicity compared to the 50 nM control neuron treatment (unpublished observations). It is not surprising that primary cells are more sensitive to drug treatment than cell lines, and along with the small therapeutic window of this drug in mice, limits the ability for interpretation of results.

That manipulation of CCR5 affects the downstream effects of both gp120 and Tat is interesting and exciting. M-tropic gp120 activates CCR5 directly by binding to the receptor promoting response in both neurons and glia (Kaul et al., 2007; Bachis et al., 2010), though very few studies have examined M-tropic gp120, the majority are interested in T-tropic gp120. However, the ways in which Tat might interact with CCR5 are less certain. Tat has been reported previously to be a chemokine mimic, as it

contains the CC motif along with other conserved residues of the  $\beta$ -chemokine family of ligands, and thus has the potential for direct activation of chemokine ligands (Albini et al., 1998; de et al., 2000) and which appears to be responsible for its neurotoxic actions (Ranga et al., 2004). Additionally Tat was able to cause chemotaxis of immune cells in a CCR3 dependent manner. While the chemotactic response was limited to CCR3 and did not include CCR5, these studies were in a different cell type and the ability of Tat to bind directly to CCR5 should not be dismissed. However, Tat is a promiscuous and sticky protein, interacting with a number of partners some of which have been identified such as CXCR4 (Xiao et al., 2000; Ghezzi et al., 2000), LDLRP (Liu et al., 2000), HSPR (Chang et al., 1997), PIP5K (Tryoen-Toth et al., 2013), integrin receptors (Urbinati et al., 2005), and the mannose receptor (Pope and Haase, 2003) while more are sure to arise from further study. Thus the potential for Tat's direct interactions, which have not been examined directly in my studies, should not overwhelm the potential for indirect activation of CCR5 by Tat through its many signaling pathways. Maraviroc was not able to modify the neurotoxic response of Tat alone, suggests that indirect mechanisms are the likely players in this model. HIV-1 Tat, in our hands, is a strong activator of glial inflammatory signaling cascades. Our studies were specific to astroglial effects, and showed that a wide range of cytokines/chemokines are upregulated by Tat treatment (El-Hage et al., 2005), including the  $\beta$ -chemokine agonists of CCR5 as well as TNF- $\alpha$ , which seems to act as a precursor to the secretion of CCR5 ligands. These studies are supported by evidence from Martin E. Dorf who examined the sequence and temporal aspects of the astroglial inflammatory signaling response (Luo et al., 2003; Luo et al., 2002). While our studies were specific to astroglia, microglial activation by Tat produces

increased CCR5 expression and interactive activation with combined morphine and Tat (Bokhari et al., 2009). And while not examined in these studies, CCR5-mediated responses have been shown to occur in oligodendroglial precursor cells as well (OPCs) which specifically upregulate  $\beta$ -chemokines when exposed to Tat and can cause microglial chemotaxis to their conditioned medium (Hahn et al., 2010). An in vivo study of the importance of the CCR5 ligand CCL5/RANTES showed that Tat and morphine mediated astroglial and microglial activation was reversed in mice lacking CCL5, suggesting that the  $\beta$ -chemokine system, and CCR5-CCL5 relationship in particular may be an important aspect of glial activation and downstream inflammatory signaling (El-Hage et al., 2008a). These studies along with the studies that were performed with maraviroc and/or CCR5 selective knockdown suggest that CCR5 may act as a downstream convergence point of both Tat and morphine signals. While the mechanism is not yet known, CCR5's ability to integrate the signals and to do so across multiple cell types speaks to its potential utility as a therapeutic drug target.

While the behavioral studies were difficult to interpret, and require more work to set up a usable model, we were able to identify certain changes in behavior in Tat-transgenic mice alone as well as interactions between genotype and treatment which may provide the starting point for manipulation of the Tat-transgenic mouse system to test proteins of interest. Motor impairments were noted in response to Tat in measures of gait analysis after three months of treatment, while one month of DOX induction coupled to morphine injections were able to alter the locomotor activating effect of morphine in Tat<sup>+</sup> mice. Other studies of motor behavior using rotarod performance as a measure also noted deficits after 3 weeks of DOX induction (Fitting et al., 2012). The

three-month DOX induction paradigm, while able to induce a wide range of behavioral changes which mimic symptoms seen in patients with HAND such as altered gait, a decrease in grip strength, and a decrease in locomotor activity which were noted in my studies or studies performed in our lab (Hahn et al., 2013), but seem to show confounding results when combined with activators of the mesolimbic dopamine pathway including morphine treatment and ICSS (Podhaizer and Miller unpublished observations). And while it is interesting to show that morphine can uncover deficits in tat-transgenic mice, as it is known that HIV-1 itself leads to myriad problems on its own, the best model may be one in which opioid treatment can exacerbate an already altered behavior, though both are important to study. Use of rotarod performance and locomotor activity tests after approximately one month of DOX induction and under various morphine paradigms may be the best way to examine interactions of morphine and HIV-1 Tat on motor behaviors with our model.

While our behavioral models were restricted to use of HIV-1 Tat, models of gp120 transgenic mouse models also exist in which gp120<sub>IIIB</sub> is induced under control of the GFAP promotor to cause brain induction of the protein (Toggas et al., 1994). This model was also able to shown neuropathological changes similar to the effects of HAND such including loss of dendritic arborization (Toggas et al., 1994;Kang et al., 2010) and changes to hippocampal-related effects including altered water maze functions, i.e. reduced escape latency and spatial retention (D'hooge et al., 1999) and reduced short- and long-term potentiation in electrophysiological measures (Krucker et al., 1998). However, few studies thus far have reported combining gp120<sub>IIIB</sub>-induction with drug administration, and those that were performed used methamphetamine (Roberts et al.,

2010;Henry et al., 2013). This model and studies provide important findings, but are not usable for the study of CCR5, as there is no model causing induction of an R5-tropic virus. Additionally, this model uses a conditional transgenic approach which does not require doxycycline induction. This is positive in terms of preventing additional effect of doxycycline treatment from influencing the results, but carries the negative effect of gp120 production throughout development. This approach takes months and sometimes over one year for effects to develop, so it is also very time consuming.

Other research in our laboratory has taken a different angle on the study of CCR5, examining the effects of maraviroc treatment in the presence of R5-tropic HIV-1 virus in the presence or absence of morphine, the results of which vary from measures of bystander effects of viral proteins (El-Hage et al., 2013). In a model of host cell infectivity, maraviroc blocked viral entry of an R5-utilizing strain, which was expected, however; surprisingly, the addition of morphine to the virus prevented maraviroc's viral entry inhibitor actions. This suggests that the mechanisms through which morphine acts to alter the inflammatory state of CNS cells are different from those involved in direct actions of virus with host, and implies that care be taken when prescribing maraviroc to cART patients. Another angle that was used to study CCR5-MOR interactions more directly was undertaken through use of a novel bivalent ligand designed by Dr. Yan Zhang at Virginia Commonwealth University. He combined two pharmacophores: one was the CCR5 non-competitive antagonist, maraviroc, and the other the opioid receptor antagonist, naltrexone, which possesses high selectivity for MOR compared to DOR and KOR (Yuan et al., 2013). These two compounds are linked by a 21 atom spacer, which separates the two pharmacophores by the ideal distance for when said

compounds are inhibitors (Daniels et al., 2005b;Daniels et al., 2005a). Bivalent ligands are currently being created to confirm the presence of putative GPCR dimers (Bhushan et al., 2004;Mathews et al., 2005;Berque-Bestel et al., 2008) and are attaining clinical applicability for the treatment of disease states (Li et al., 2010). In contrast to the findings that in a model of cell infectivity, maraviroc's protective actions were blocked in the presence of morphine, the bivalent ligand acted to reverse the pro-infectivity of morphine and did so to a significantly greater extent than did the pharmacophores that were applied separately. Interestingly though, this powerful effect of the bivalent ligand only occurred in astrocytes, and was not present in microglia. The studies suggest that both the low level of MOR in the microglial cells tested and the paucity of MOR/CCR5 co-expressing cells compared to that in astrocytes may be the reason for the lack of response in this cell type, which is actually responsible for the majority of the infectivity in the brain.

Treatment with maraviroc, or other CCR5 antagonists/receptor modifiers may be able to 1) block viral entry and infection of R5-tropic HIV-1 viruses, 2) attenuate the actions of toxic HIV-1 viral proteins alone, and 3) disrupt the pathways involved in opioid-HIV-1 interactive neurotoxicity and neuron damage. However, based on the findings thus far, for continued use of maraviroc as a component of cART, care should be taken in those who receive it as part of their regimen, and people should be assessed for their co-morbid use of injection drugs, such as opioids. Currently use is restricted to those who are currently cART experience and those who only have R5 virus (Youngblood et al., 2011). It appears that waiting to introduce maraviroc into cART therapy should be continued until viral load is controlled in those abusing opioids.



However, after this point, maraviroc may be beneficial to prevent CNS inflammation and control neurocognitive impairments, though more studies need to be done to confirm this.

Additionally, study of the bivalent ligand should be extended to studies of neurodegeneration in addition to determine if this type of inhibitor has improved performance without inhibition by the presence of morphine. This may be impetus for a new line of therapy in the opioid abusing, HIV-1 infected populations. While the current bivalent ligand does not function well in microglia compared to astroglia, it may not have good utility in reducing viral entry and infection, but may still be useful in preventing HIV-1-induced inflammation especially in the presence of opioids and at a greater efficacy than maraviroc alone.

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## Vita

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